Memories of the soils:

Evaluation of soil nitrogen stable isotope as a robust metric to assess floodplain restoration and nitrogen removal effectiveness

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Executive Summary

Stream restoration is a billion-dollar industry in the United States, with stream and floodplain restorations being prolific throughout the Mid-Atlantic for over 20 years. Though numerous projects have been completed (many to meet 303d the Total Maximum Daily Load (TMDL) limits), long term monitoring of restorations is largely lacking. It is often proposed that denitrification will increase after stream restoration and floodplain reconnection, but with little post-construction monitoring, this largely remains to be seen. The main goal of our research was to determine if $\delta\,^{15}N$, a soil nitrogen (N) stable isotope, could be used as a proxy for microbial denitrification. Soils with elevated denitrification rates are expected to have elevated or enriched soil $\delta\,^{15}N$ values. Thus, the $\delta\,^{15}N$ soil metric could be a robust, integrated metric for denitrification and N removal effectiveness for restored floodplains. Other metrics including - Total Organic Carbon (TOC), organic matter, bulk density, soil moisture and particle size distribution were measured and compared to denitrification rates to determine if they co-varied as well.

Twelve sampling sites were selected based on the date of restoration completion and grouped into four different age categories, 0-2 years old, 2-5 years old, 5-10 years old and 10-22 years old (3 sites each). We collected mineral soil, bulk density, and water samples from the restored sites across Maryland and Virginia between April 18th and May 10th, 2023. Both restored and unrestored sections of the floodplain were sampled to assess the effects of restoration on floodplain soil and water.

Results showed a general decline in $\delta^{15}N$ over time at restored sites which was confirmed by multiple analyses including t-tests and ANOVAs. This result was contrary to what we hypothesized - increased soil $\delta\,^{15}N$ post restoration due to enhanced denitrification enrichment of

δ15N. We attribute this result to increased vegetation and tree growth in the restored floodplain post restoration that increased depleted δ^{15}N organic N inputs to the soils. Initially, δ^{15}N and denitrification rate (amended and unamended) were positively correlated for the 0-2 year age category. After that, correlation intensity decreased and the correlation changed from positive to negative.

TOC, organic matter, and soil moisture were all significantly positively related to denitrification while bulk density was significantly negatively correlated. These parameters exhibited the most significant relationships at the 10-22 year age category but varied consistently over time. These metrics indicate that while δ^{15}N may not be the robust restoration metric for denitrification assessment, stream restoration was successful in improving the floodplain environment for these metrics.

Introduction

Anthropogenic activities such as agriculture and urbanization and their legacies have significantly altered the geomorphic structure, flow regime, and water quality of streams and rivers across the United States (US) and worldwide (Carpenter et al., 1998; Galloway et al., 2004; Inamdar et al., 2021; Walter and Merritts, 2008). This has resulted in impaired streams with flashy flows, elevated sediment and nutrient concentrations, and poor ecological habitat (Forshay et al., 2022; Kaushal et al., 2008; Mayer et al., 2022). Stream restorations seek to remedy these conditions by reconnecting stream channels with their floodplains, decrease streamflow velocities and enhance infiltration, reduce peak discharges and sediment loads, and provide a healthy, carbon-rich soil environment for biogeochemical processes to remove of excess nutrients (Berg, 2014; Inamdar et al., 2023; Lammers and Bledsoe, 2017; Mayer et al., 2022; Wohl et al., 2015). These restorations are accomplished through raising stream beds, introduction of channel meanders, pools and riffles, removal and/or grading of streambank sediments, and establishment of native riparian or wetland revegetation on the restored floodplain (Wood and Schueler, 2020).

Stream and floodplain restorations have especially become popular in the US with more than a billion dollars spent annually nationwide (Bernhardt et al., 2005) and 400 million being spent since 1990 in the Chesapeake Bay watershed alone (Hassett et al. (2005). Similarly, the number of stream restorations has grown exponentially every decade (Bernhardt et al., 2005), and the average restoration length has almost tripled, from 233 to 670 m (Dance, 2020). These changes suggest that stream restorations are becoming more popular, more studied, and larger in their footprint. Many of the streams targeted for restoration are listed on the state’s total maximum daily load (TMDL) listing of impaired water bodies. Thus, sediment and nutrient improvements/gains from restoration are being used as credits to meet water quality regulatory (TMDL) targets (Berg, 2014; Mattern et al., 2020).

While these restoration efforts have provided important gains towards enhancing stream, floodplain, and ecosystem health, many questions and concerns remain about the long-term effectiveness of these restorations for improving water quality (Bernhardt and Palmer, 2011; Dance, 2020; Palmer et al., 2014). Typically, restorations implement the beneficial conditions for stream and floodplain hydrology, vegetation, and soil type with the implicit assumption that nutrient removal functions and benefits will follow automatically. In essence, the projects follow the “Field of Dreams” paradigm of “if you build it, they will come”. Whether this happens or not is an open question which is not being addressed.

A key reason for the knowledge gap on effectiveness or performance of the restoration projects is that many of the stream restoration projects have little monitoring component, pre- or post-restoration, and there is limited information to determine if the restorations achieved their water quality objectives (Palmer et al., 2014). For example, Hassett et al. (2005) reported that only 5.4% of the 4700 projects in their synthesis of studies in the Chesapeake Bay watershed report monitoring. This lack of monitoring has been because of (Buchanan et al., 2014): (a) unavailability of funding and resources; (b) lack of regulatory requirements; (c) absence of reliable and robust metrics for assessment, particularly long term; and (d) natural variability in system response because of spatial and temporal (e.g., baseflow versus stormflows) differences and climate/weather variations (Filoso and Palmer, 2011; Williams et al., 2017). If we are to enhance the effectiveness and resilience of these restorations, increase the return on our large investments, and address stakeholder and media concerns (e.g., Cox, 2018; Dance 2020), we need to monitor the sites post restoration and develop robust metrics for monitoring.

Denitrification is a key ecosystem service provided by stream and floodplain restoration that is central to nitrogen removal (Kaushal et al., 2008; Mayer et al., 2022; Vidon et al., 2010). Denitrification converts reactive nitrate-N in runoff waters to inert N2 gas and thus reduces nitrogen pollution. Frequent inundation of floodplain soils via overbank flooding and floodplain reconnection is beneficial for denitrification as this provides the necessary soil moisture and anoxic environment for the process (Mulholland et al., 2009; Roley, Tank, and Williams, 2012; Vidon et al., 2010). Similarly, increased organic carbon inputs from restored riparian vegetation provides the energy that enhances denitrification (Gift et al., 2008; Mayer et al., 2010; Newcomer et al., 2012).

Previous restoration monitoring studies have typically assessed short term N concentration changes in surface and groundwaters and only a few have measured denitrification rates and identified their key drivers in floodplain soils over long durations (Forshay et al., 2022; Newcomer-Johnson et al., 2014; Mayer et al, 2022; Napora et al., 2023). Napora et al. (2023) studying a 1-10+ year chronosequence of restored floodplains found that total carbon and nitrogen in floodplain soils increased over time and attributed it to increased soil wetness and organic matter content. Forshay et al. (2022) found that floodplain soil denitrification increased over a five-year period and suggested that increased accumulation of soil organic carbon was a key driver.

Similarly, bulk soil δ^{15}N has also been used as a proxy for microbial denitrification (Hasselquist et al., 2017; O'Neill et al., 2021; Roberts et al., 2023; Yang and Toor, 2016). δ15N represents the ratio of heavier ^{15}N to lighter ^{14}N isotopes and can be used to provide an integrated response of multiple N processes in soils (Bedard-Haughn et al., 2003; Nestler et al., 2011). Processes that preferentially remove ^{14}N enrich or increase δ^{15}N while processes that add ^{14}N deplete or decrease $\delta^{15}N$ (referred to as fractionation). Denitrification has a strong enrichment effect on $\delta^{15}N$ due to preferential conversion of ^{14}N molecules to N_2 gas, while processes like nitrification deplete $\delta^{15}N$ via addition of ^{14}N. Studying a 25-year chronosequence of 17 restored sites along Boreal riparian floodplains in Sweden, Hasselquist et al. (2017) found that foliage and soil $\delta^{15}N$ initially increased after restoration but then declined thereafter to more stable values. They attributed the initial increase in $\delta^{15}N$ to denitrification enrichment which itself was stimulated by the short term pulse increase in N due to soil disturbance and associated mineralization and nitrification of N. The decline in $\delta^{15}N$ thereafter during the "reorganization" restoration phase was attributed to decrease in microbial processing and lack of N availability. Others have also suggested that the soil and foliage $\delta^{15}N$ response post restoration could be complex and could be affected by multiple drivers including inputs from various N sources (atmospheric deposition, fertilizers, manure, etc.), mycorrhizal fungi and their role in N uptake by plants, and changes in riparian vegetation species post restoration, particularly N fixers that can increase availability of ^{14}N (Bai et al., 2013; Hobbie and Oiumette, 2009).

Our interest here was to advance our understanding of denitrification and associated soil properties (bulk density, organic matter, soil moisture and total soil C and N pools), including $\delta^{15}N$, for floodplain soils over the long term. We investigated 12 restored floodplain sites in the Mid-Atlantic US that spanned a chronosequence of 0-25 years. Sampling was performed for unrestored and restored reaches of the sites and for two "reference" sites that were undisturbed. Key questions that we addressed were: How do denitrification, soil bulk $\delta^{15}N$ and soil properties change following restoration? Are any of the soil parameters more sensitive and provide a robust proxy for denitrification post restoration? Specific hypotheses associated with the questions were:

- **H1**: δ^{15}N is strongly and positively correlated with floodplain soil denitrification.
- **H2**: Unrestored, disconnected floodplain has low denitrification, more depleted ^{14}N, and thus low δ^{15}N compared to restored and well-connected floodplain.
- **H3**: Immediately after restoration (0-2 yrs) δ^{15}N will be highly variable because of restoration-related disturbance effects.
- **H4**: Post restoration: Increased denitrification after restoration removes lighter ^{14}N via denitrification and increases δ^{15}N. But, as floodplains age (> 20 yrs), δ^{15}N decreases slightly as N availability decreases.

BOX 1: Definition of δ^{15}N: Nitrogen is composed of two naturally occurring stable isotopes of atomic masses 14 and 15, with the lighter isotope (^{14}N) composing a majority (99.6337%) of the N (Kendall and McDonnell, 1998). While the ^{15}N isotope fraction is small, it can be quantified, and is highly responsive to select N cycle processes (Craine et al., 2015, Kendall and McDonnell, 1998). Thus, it is a sensitive metric that provides an integrated response of various N cycle processes in ecosystems (Glibert et al., 2019). The natural ^{15}N abundance is typically expressed as delta δ^{15}N (per thousand or mil ‰) (Bedard-Haughn et al., 2003):

$$\delta^{15}\text{N (‰)} = \left[\frac{R_{sample} - R_{standard}}{R_{standard}}\right] \text{x } 1000$$

Where R_{sample} and $R_{standard}$ are the molar ratios of ^{15}N to ^{14}N in the sample and the standard, respectively. The lighter ^{14}N molecule is energetically easier to consume or remove and is thus preferentially lost during N cycle processes. This results in - fractionation - increasing amounts of ^{15}N (enrichment of δ^{15}N) in the substrate and a higher proportion of the lighter ^{14}N in the product (depletion of δ^{15}N). N cycle processes that enhance this fractionation include microbial processes such as denitrification and abiotic processes such a volatilization. Denitrification, in particular, has been shown to be strongly fractionating by increasing the δ^{15}N content of water (Bedard-Haughn et al., 2003; Sebilo et al., 2003) and soils in wet, reducing environments (Bedard-Haughn et al., 2003; Craine et al., 2015). δ^{15}N enrichment due to nitrification is minimal in soils with low ammonium-N (Kendall et al., 2007).

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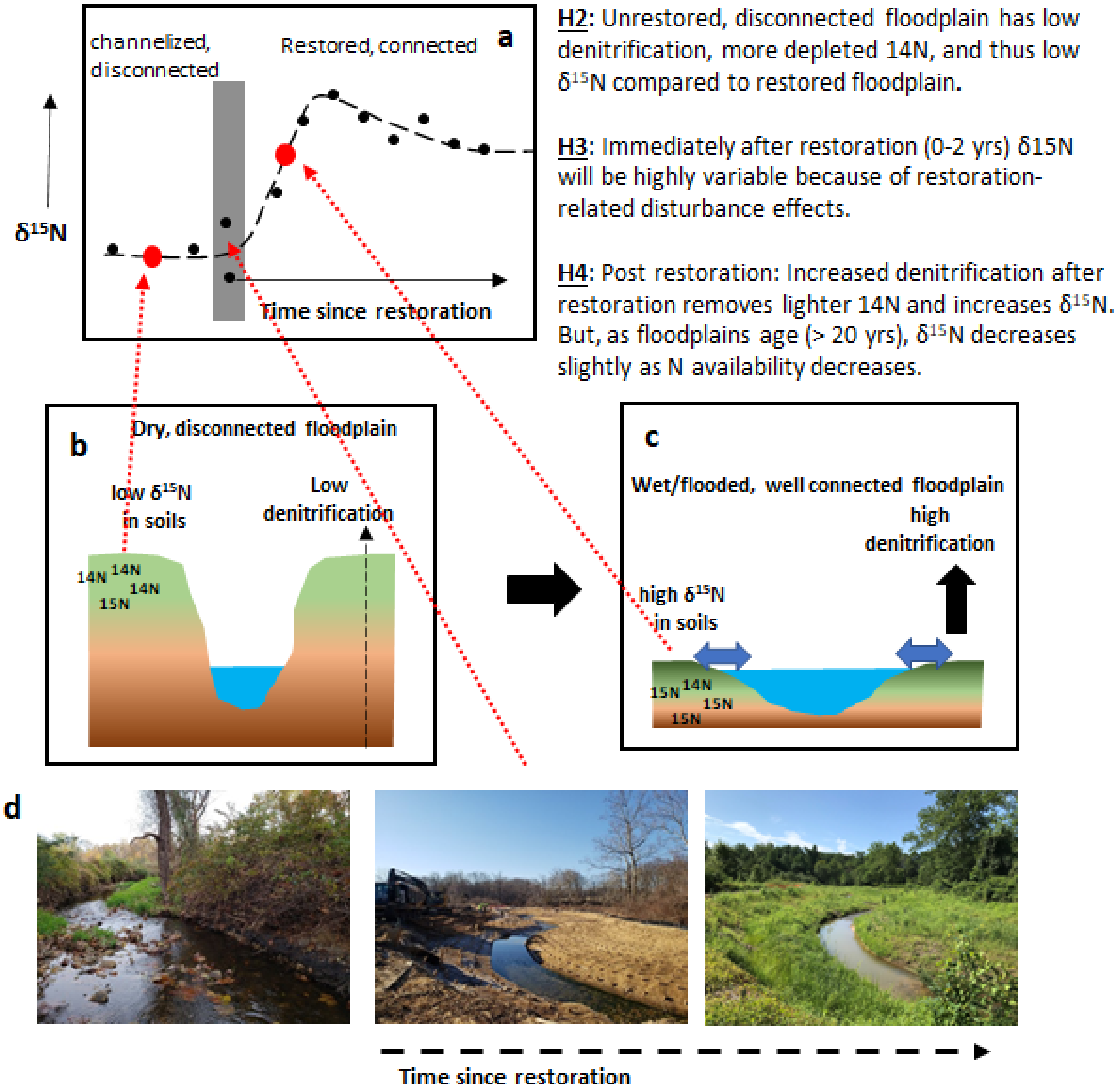
HYPOTHESES:

H1: $\delta^{15}N$ is strongly and positively correlated with floodplain soil denitrification.

H2: Unrestored, disconnected floodplain has low denitrification, more depleted 14N, and thus low $\delta^{15}N$ compared to restored floodplain.

H3: Immediately after restoration (0-2 yrs) δ15N will be highly variable because of restoration-related disturbance effects.

H4: Post restoration: Increased denitrification after restoration removes lighter 14N and increases $\delta^{15}N$. But, as floodplains age (> 20 yrs), $\delta^{15}N$ decreases slightly as N availability decreases.



Box 2: Conceptual model illustrating the changes in soil $\delta^{15}N$ as floodplain denitrification increases following restoration. The graph (**a**) indicates the hypothesized temporal trajectory of soil $\delta^{15}N$ from pre to post restoration. Hypotheses are listed on top right. The cross-sections (**b and c**) indicate the floodplain height and hydrologic (dis)connection and effects on denitrification. The photos (**d**) are from the Gramies Run legacy sediment removal and stream-floodplain restoration that was completed in 2019 in Cecil County, Maryland.

In order to assess the effect that stream restoration age has on denitrification and associated bulk soil $\delta^{15}N$, 12 different restored streams located throughout the Mid-Atlantic were identified for sample collection. These sites mainly used the Natural Channel Design (NCD) of restoration methodology. Notable additions to this approach include the restoration at Broad Creek which used both Step Pool Storm Conveyance (SPSC) and NCD based valley restoration techniques and Howards Branch which employed a floodplain reconnection approach using organic rich mulch. Sites were grouped into four separate age categories 0-2, 2-5, 5-10, and 10-22 years since restoration was conducted. Sites were evenly divided amongst the age categories meaning each age bracket contains three sites.

Sites and Methods

Sampling sites (Table 1) include Broad Creek, Kelly Branch, Stoney Run, Grammies Run, Scotts Level Branch (at Morriottsville Rd.), Scotts Level Branch (at McDonogh Rd.), Bacon Ridge, Difficult Run, Catonsville Park, Minebank Run, Spring Branch, and Howards Branch (Figure 1).

Table 1: The 12 floodplain restoration sites selected for this study. Sites are divided into four separate age groups 0-2 years, 2-5 years, 5-10 years, and 10-22 years. The exact coordinates of the restoration are provided along with its state name.

Site	**Age Range (Years)**	**Date Restored**	**State**	**Latitude / Longitude**
Broad Creek	0-2	2021	MD	38.969415°, -76.568360°
Kelly Branch	0-2	2021	MD	39.442490°, -76.592562°
Stoney Run	0-2	2022	MD	39.633255°, -75.964033°
Gramies Run	2-5	2020	MD	39.686514°, -75.850506°
Scotts Level Branch at Morriottsville Rd	2-5	2019	MD	39.383717°, -76.819942°
Bacon Ridge	2-5	2020	MD	39.037240°, -76.627585°
Difficult Run	5-10	2014	VA	38.933449°, -77.338004°
Catonsville Park	5-10	2017	MD	39.279201°, -76.750729°
Scotts Level Branch at McDonogh Rd	5-10	2014	MD	39.374473°, -76.792333°
Minebank Run	10-22	2005	MD	39.416075°, -76.547572°
Spring Branch	10-22	2008	MD	39.440247°, -76.597526°
Howards Branch	10-22	2001	MD	39.021048°, -76.548883°

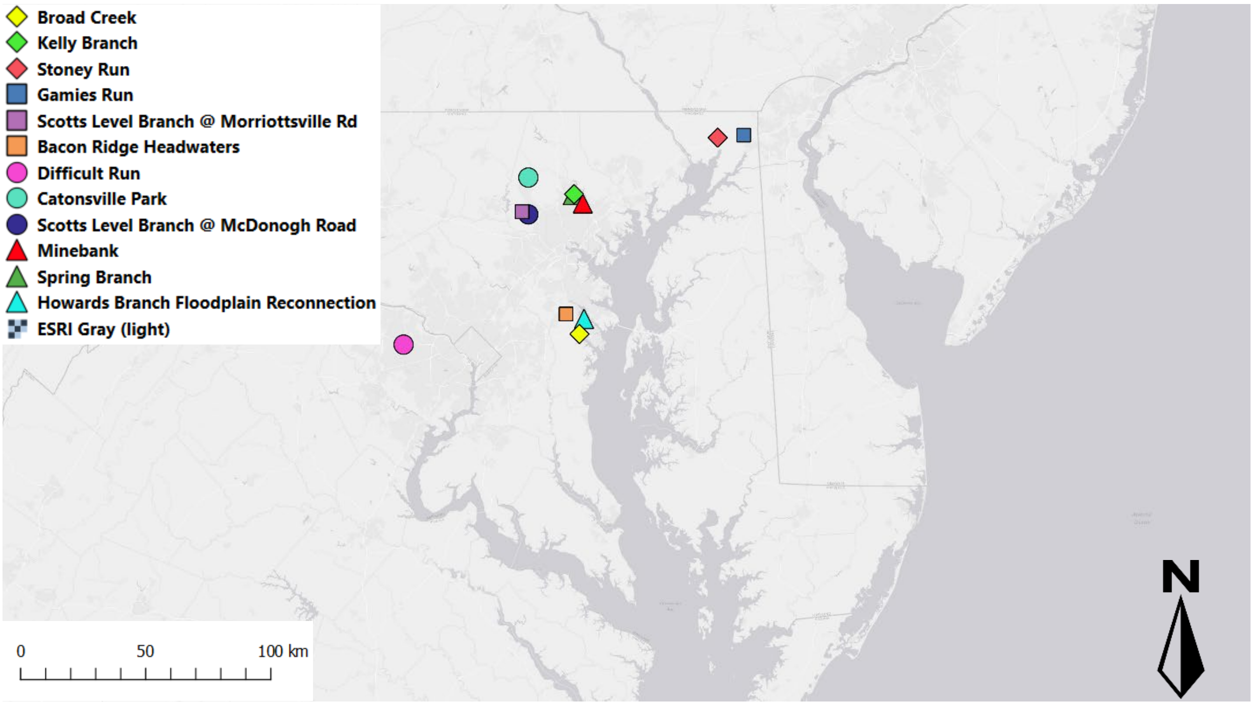


Figure 1: Locations of the 12 sampling sites selected for this study. Sites are all within the Chesapeake Bay watershed and have had a floodplain restoration conducted within the past 22 years.

Field sampling:

Soil and water samples were collected from the floodplain sites between April 18 and May 10, 2023. A shovel was used to remove brush and foliage from the surface down to ~2 inches of depth. Soil samples were collected via a shovel within the top 8 inches of soil depth and stored in labeled one quart zip-top bags. Ten samples were collected from each restoration site (Figure 2). Six samples were collected from restored reaches, three from each bank at 1, 2, and 3 meters from the bank. Four samples were collected from unrestored reaches, two from each bank at 1 and 3 meters from the bank. Water samples were collected in acid washed 250 ml Nalgene bottles from approximately the transition point between an unrestored and restored reach. Soil and water samples were transported in a cooler with ice and kept in a lab refrigerator. Water samples were filtered and acidified with ultra-pure HCL until a pH of <2 was achieved.

At all 12 sampling sites bulk density analysis was also completed. Sampling was conducted using metal sampling rings with a known length and inner diameter. Sample collection was done after the top 2-3 inches of brush had been cleared by tapping the pipe into the soil using a shovel. This methodology was used to prevent unnecessary compaction of the soil which would alter results. Excess soil was removed from the sample ring using a pocketknife and the sample was stored in a sealed zip-top bag for later analysis.

Figure 2: Joe Galella (left) and Moklesur Rahman (right) collecting floodplain soil samples from the recently restored section of the Stoney Run creek in Cecil County, Maryland.

Laboratory Analysis:

Bulk density masses were measured in grams with calibrated high precision lab scales. Samples were dried in a lab oven set to 65º C for 48 hours and the dry mass of the soil was determined. Bulk density was determined by dividing the soils dry mass by the measured volume of each sampling ring. Soil bulk density measurements are recorded in g/cm^3. Soil porosity was calculated using the formula ((1 - (Bulk Density ÷ Particle Density)) x 100) where particle density was assumed to be 2.65 g/cm^3. Gravimetric Water Content (GWC) was calculated by dividing the water mass by the soil mass of each sample. Volumetric Water Content (VWC) was calculated by dividing the volume of water by the volume of soil in each sample.

Soil ammonium (NH_4) concentrations were determined using potassium chloride (KCl) extraction. NH_4 was extracted from two grams of soil using 20 mL of KCl solution (2 N), shaken filtered and processed. A Lachat flow injection analysis system was used for final analysis.

Total organic carbon (TOC) concentrations were determined using a one-gram soil aliquot treated with one ml of sulfurous acid (6%). All carbonate (inorganic carbon) was removed by the sulfurous acid, leaving only organic carbon remaining in the sample. A LECO brand combustion analysis instrument was used for sample analysis. A resistance furnace (set to 1350˚C) is used to ignite the sample in an oxygen rich environment and an aliquot of the combustion gas is analyzed via an infrared absorption detector to determine organic carbon content.

Total Nitrogen (TN) concentrations were determined using a one-gram soil sample. The sample is put in a combustion boat and analyzed via a LECO brand combustion analysis instrument set to 1350˚C. Resulting combustion gasses are analyzed for N content.

Soil texture (% sand, silt and clay) was determined via hydrometer analysis. A 50 g soil sample aliquot was mixed into a deionized water (1L) and sodium hexametaphosphate (50 g) solution using a blender for four minutes. Hydrometer and temperature readings are recorded after inverting the sample cylinder at 40 seconds and then once again after two hours. If needed, samples with high organic matter (> 6%) are pretreated with hydrogen peroxide (30%) in order to promote dispersion and disaggregation of microaggregates. Amyl alcohol was used to disperse any foam present.

Denitrification Enzyme Activity (DEA) Assays were conducted using gas chromatographic (GC) analysis to measure N_2O gas concentrations. The purpose of DEA analysis is to measure the expression of denitrification enzymes which are present in soil or sediment at the time of collection. Five-gram soil samples (+/-0.5g) were placed into 125 ml Erlenmeyer flasks with 10 ml of DEA Media. This DEA solution was added to each individual sample before incubation in order to supply denitrifying bacteria with a source of organic carbon and NO3 to prevent substrate limitation. The solution also contains chloramphenicol to inhibit new enzyme synthesis. DEA media was prepared according to (Table 2) below.

Table 2: DEA media composition by quantity required (Chloramphenicol inhibits protein synthesis; for unamended DEA, media with only chloramphenicol was used).

DEA Media:

200 ml media	500 ml media	1L media	2L media
0.144g KNO_3	0.36g KNO_3	0.72g KNO_3	1.44g KNO_3
0.10g glucose	0.25g glucose	0.50g glucose	1.00g glucose
0.025g Chloramphenicol	0.0625g Chloramphenicol	0.125g Chloramphenicol	0.250g Chloramphenicol
MQ to 200 ml	MQ to 500 ml	MQ to 1L	MQ to 2L

A solution containing only chloramphenicol was used for measuring unamended denitrification. To ensure an anoxic environment during the incubation, the samples were flushed helium (He) gas to remove any oxygen present. Flushing was conducted for 1 minute and repeated three times for each sample. Ultra-pure acetylene gas was used to inhibit the reduction of N_2O during the incubation period. After shaking for 30 minutes and 60 minutes, aliquots were analyzed via GC using a 63Ni electron capture detector at 350ºC which measured the electron absorption of each sample.

$\delta^{15}N$ and $\delta^{13}C$ analysis was conducted on aliquot soil samples dried in a lab oven at 60ºC for 24 hours and ground with a mortar and pestle. A Nc2500 Elemental Analyzer (EA) and a Thermo Delta V+ Mass Spectrometer (IRMS) were used in conjunction for sample analysis. Samples were combusted in an oxygenated column (at 900°C) filled with silver cobaltous and chromium oxide. Sample gases (as well as helium carrier gas) flowed into a reduction column (at 650°C) filled with copper granules which converted the N oxides present into molecular nitrogen (N2). CO_2, N_2, and water vapor were diverted through a magnesium perchlorate water trap and a GC column (at 50°C) to assist in peak separation before entering the IRMS.

The composition of samples were matched with a quality control standard (made in-house at UMCES), which were analyzed alongside the samples. In-house standards were previously calibrated against commercially available external standards. Two different known standards (USGS 40 and USGS 41 or house-made standards that have been calibrated against the commercially available standards) were analyzed every time the instrument runs to normalize values for each batch of samples. A single blank was also included in each analysis to detect background CO_2 or N_2. Every analysis had an initial acetanilide "bypass" sample (~1mg) which acted to prime the system and to check for any irregularities. Three samples of atropine were then analyzed in a calculated range of weights in order to obtain %C and %N data. The long-term standard deviation (1σ) of internal standards were 0.12‰ for $\delta^{13}C$ and 0.11‰ for $\delta^{15}N$. If the $\delta^{13}C$ and $\delta^{15}N$ values for more than one of the internal QC samples in a given sample set exceeds the 2σ range of the known value for that QC material then the samples are re-run. The final isotopic values are expressed relative to external standards V-PDB (Vienna PeeDee Belemnite) and Air for carbon and N, respectively.

Stream water $\delta^{18}O\text{-}NO_3^-$ and $\delta^{15}N\text{-}NO_3^-$ concentrations were determined via using titanium trichloride reduction and trace gas preconcentrator. The methodology used followed the Altabet et al., methodology proposed in "A Ti(III) reduction for one-step conversion of seawater and freshwater nitrate into N_2O for stable isotopic analysis of $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{17}O/^{16}O$" (Altabet *et al.*, 2019).

Statistical Methods:

For each sampling site 10 data points were available – 6 from the restored site (at 1, 2, and 3 m from the stream edge on the left and the right bank and 4 from the unrestored reach (at 1 and 3 m from the stream edge from the left and the right bank). ANOVA was also performed for samples collected at 1, 2, and 3m from the stream edge for the restored sites. Since the data were not significantly different the data was lumped together for comparisons between age categories and sites. When comparisons or correlations were performed between restored and unrestored sites within an age category (3 sites) there were 12 data points (4 x 3) while for restores sites there were 18 data points (6 x 3).

Pearson correlation coefficient was used to determine the strength of linear relationships. Results vary -1 to 1 depending on strength and direction of relationship between two variables with -1 representing a perfect negative linear relationship and 1 representing a perfect positive linear relationship.

Significant values (p value <0.05) are bold and denoted with a *. Prior to analysis the distribution of the data was checked for normality. If the data were not normally distributed they were log transformed.

Spearman correlation coefficient was used to determine the strength of nonparametric measure of rank correlation. Results vary -1 to 1 depending on strength and direction of relationship between two variables with -1 representing a perfect negative nonparametric relationship and 1 representing a perfect positive nonparametric relationship. Significant values (p value <0.05) are bold and denoted with a *.

One-way analysis of variance (ANOVA) and two-way ANOVA were used for hypothesis testing. One way ANOVA determines whether the means of two samples are significantly different (using an F distribution). One-way ANOVA analysis requires one numeric response variable "Y" and one explanatory variable "X". Two-way ANOVA analysis functions similarly to the one-way ANOVA but examines the effect of two different independent variables (age category and restoration status) and one continuous variable (soil denitrification rate, organic matter, total organic carbon, bulk density, moisture, and $\delta^{15}N$). Two-way ANOVA determines the main effect of independent variables and any interaction between them.

Tukey-Kramer's Honestly Significant Difference (HSD) test was used to determine pair-wise comparisons among the means of respective independent variables and interactions among treatments to determine which age category and restoration status or combination thereof significantly influenced dependent variables. Data were analyzed using JMP Pro, software.

Results:

Changes in soil denitrification rates, organic matter, bulk density, and $\delta^{15}N$ with restoration age

Box and whisker plots were created for denitrification rate, organic matter, total organic carbon, bulk density, soil moisture and $\delta^{15}N$ for restored sites (Figure 3). The X axis for all plots were the age categories selected for this study with three sites being represented in each category. Denitrification showed a general positive trend, increasing with time. Variability in the dataset was notably higher in the 2-5 and 10-22 year age categories. Organic matter, total organic carbon and soil moisture all increased with restoration age as well, with the mean and maximum values being highest for all being in the 10-22 year age category. Bulk density and δ^{15}N both initially increased for restored sites for the 0-2 age category, but then decreased over time with the lowest average concentrations being at the 10-22 year age category.

Bulk density, organic carbon, organic matter, δ^{15}N, soil moisture and denitrification rate were analyzed via two-way analysis of variance (ANOVA) with age category, restoration status (unrestored and restored) and age category and restoration status being the groups tested (Table 3).

Table 3. Two-way ANOVA results for soil denitrification rate, organic matter, total organic carbon, bulk density, moisture, and δ^{15}N. Significant p-values (<0.05) are in bold. Please note that for each study site we had 10 data points (6 from restored and 4 from unrestored sites). For each age group with 3 study sites that amounted to total 30 data points (18 for restored and 12 for unrestored).

	Age Category			Restoration Status			Age Category : Status		
Variable	DF	F-ratio	p-value	DF	F-ratio	p-value	DF	F-ratio	p-value
Bulk Density g/cm^3	3	5.095	**0.0025**	1	2.811	0.0965	3	1.074	0.3635
Organic Carbon %	3	5.733	**0.0011**	1	17.402	**<0.0001**	3	2.656	0.0522
Organic Matter % Unamended	3	1.6863	0.1744	1	8.3905	**0.0046**	3	4.0417	**0.0092**
δ15N ‰	3	2.58	0.0574	1	0.0409	0.5236	3	6.094	**0.0007**
Soil Moisture % Unamended	3	0.6351	0.594	1	1.6675	0.1994	3	5.5885	**0.0013**
Denitrification Rate (ug N/kg/h)	3	10.8244	**0.0001**	1	1.689	0.1966	3	1.782	0.155

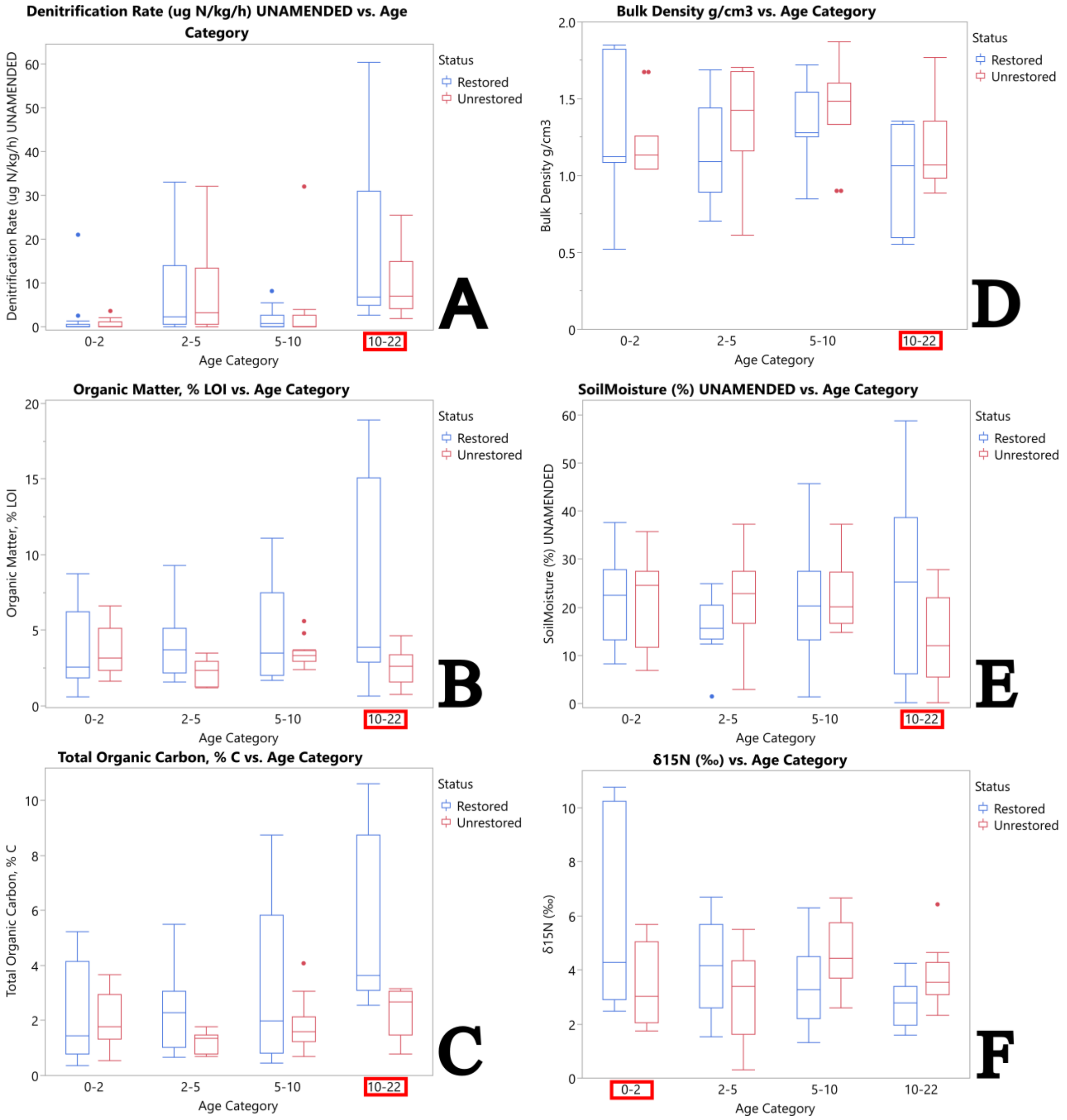


Figure 3. Box and whisker plots by age category of restored (blue) and unrestored (red) sites. Soil denitrification rate (A), organic matter (B), total organic carbon (C), bulk density (D), moisture (E), and δ^{15}N (F) at restored sites. Significantly different results (as determined through 2-way ANOVA and Tukey-Kramer HSD connecting letters) are highlighted with a red box.

Box and whisker plots for sand silt and clay composition were created to determine how they differed between restored and unrestored sites (Figure 4). Composition remained the same for all age categories whether restored or unrestored with sand making up the greatest fraction, followed by silt and then clay. At most restored sites the variability of samples was smaller than at unrestored sites.

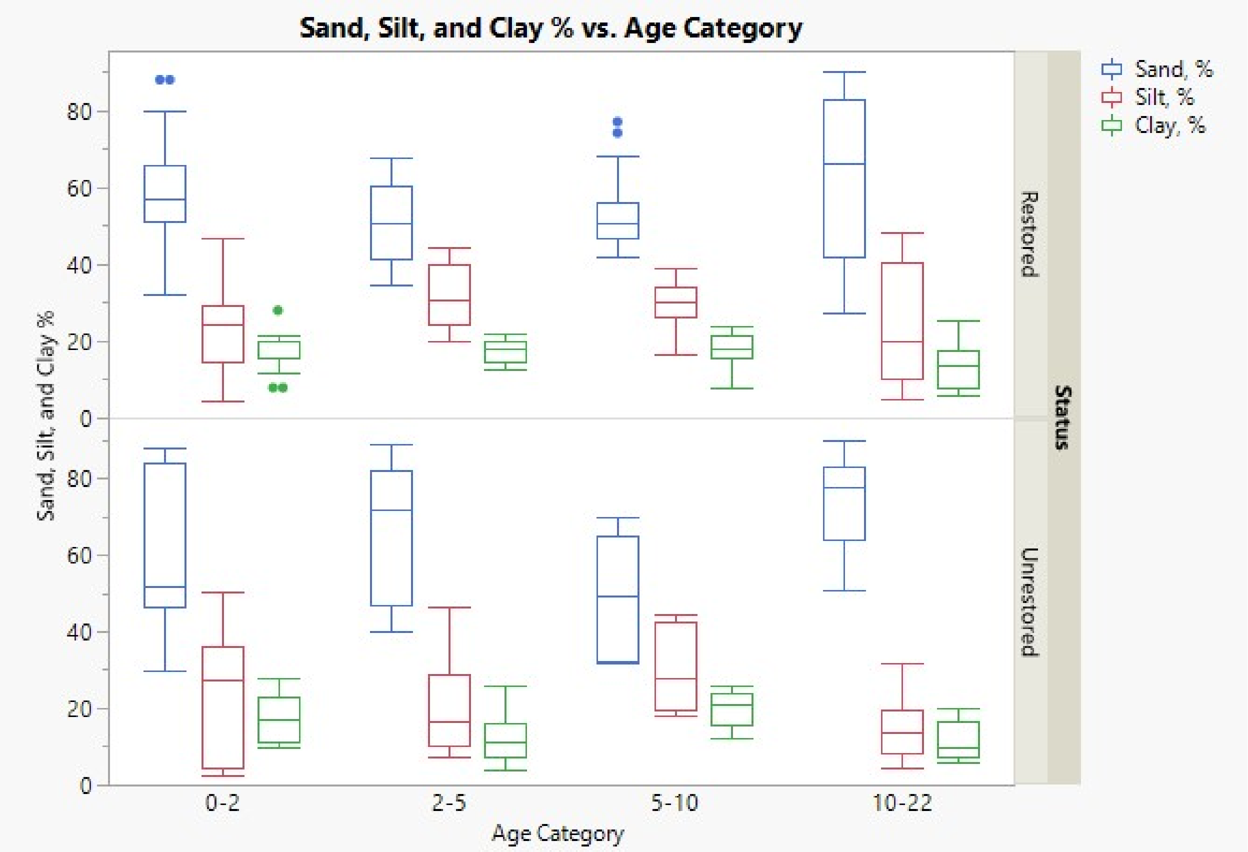


Figure 4. Box and whisker plot analysis of particle size distribution in restored and unrestored streams by age category.

Relationship between 15N and denitrification

• **H1**: δ^{15}N is strongly and positively correlated with floodplain soil denitrification.

Unamended denitrification enzyme assay results showed inconsistent correlation with δ^{15}N (Tables 4 and 5). The youngest restored sites in the 0-2 year age category showed a weak positive linear correlation (Pearson's correlation coefficient 0.4305) and a significant nonparametric positive correlation (Spearman ρ 0.6682) between denitrification rate and δ^{15}N . All other age categories of restored sites showed little to no correlation in either linear or nonparametric analysis. The direction of correlation (positive or negative) was found to alternate from positive to negative at random intervals (Table 4 and 5, and Figure 3).

Table 4: Pearson's correlation coefficient between unamended denitrification and δ^{15}N for restored and unrestored sites within each age category (-1 to 1 depending on strength and direction of relationship between variables). Measures linear correlation between two sets of data. Significant values are bold and denoted with a *. The number of data points used "n" is indicated in the Table within brackets).

Age Category	**Restored**	**Unrestored**
0-2 Years	0.4305 (n = 18)	-0.927 (n=12)
2-5 Years	-0.331 (18)	0.2346 (12)
5-10 Years	0.2150 (18)	0.1895 (12)
10-22 Years	-0.3395 (18)	-0.3833 (12)

Table 5: Spearman correlation coefficient (-1 to 1 depending on strength and direction of relationship between variables). Measures nonparametric measure of rank correlation between two sets of data. Significant values are bold and denoted with a *. The number of data points used "n" is indicated in the Table within brackets).

	Restored		**Unrestored**	
Age Category	Spearman ρ	Prob>|ρ|	Spearman ρ	Prob>|ρ|
0-2 Years	0.6682	***0.0034 (n=18)**	-0.0780	0.8096 (12)
2-5 Years	-0.3299	0.1960 (18)	0.2517	0.4299 (12)
5-10 Years	0.2916	0.2404 (18)	-0.1040	0.7477 (12)
10-22 Years	-0.2036	0.4668 (18)	-0.4091	0.2115 (12)

Amended denitrification enzyme assay results showed inconsistent positive correlation with δ^{15}N similar to unamended results. The youngest restored sites in the 0-2 year age category showed a weak positive linear correlation (Pearson's correlation coefficient 0.3899) and nonparametric positive correlation (Spearman ρ 0.4839) between denitrification rate and $\delta^{15}N$ (Table 6 and Table 7). A weak negative linear correlation is present at the 2-5 year age category as well but this is likely influenced by an outlier in the dataset. All other age categories of restored sites showed little to no correlation in either linear or nonparametric analysis. The direction of correlation (positive or negative) was found to alternate from positive to negative at random intervals (Table 6 and 7, and Figure 3).

Table 6: Pearson's correlation coefficient (-1 to 1 depending on strength and direction of relationship between variables). Measures linear correlation between two sets of data. Significant values are bold and denoted with a *.

Age Category	Restored	Unrestored
0-2 Years	0.3899	0.4860
2-5 Years	-.4081	0.5508
5-10 Years	0.1457	0.0706
10-22 Years	0.0355	0.2544

Table 7: Spearman correlation coefficient (-1 to 1 depending on strength and direction of relationship between variables). Measures nonparametric measure of rank correlation between two sets of data. Significant values are bold and denoted with a *.

	Restored		**Unrestored**	
Age Category	Spearman ρ	Prob>|ρ|	Spearman ρ	Prob>|ρ|
0-2 Years	0.4839	***0.0490**	0.5303	0.0762
2-5 Years	-0.3470	0.1723	0.4336	0.1591
5-10 Years	0.4575	0.0563	-0.2575	0.4190
10-22 Years	-0.3286	0.2318	-.1	0.7699

$\delta^{15}N$ variability in restored and unrestored sites by age category

- **H2**: Unrestored, disconnected floodplain has low denitrification, more depleted ^{14}N, and thus low $\delta^{15}N$ compared to restored and well-connected floodplain.
- **H3**: Immediately after restoration (0-2 yrs) $\delta^{15}N$ will be highly variable because of restoration-related disturbance effects.

Box and whisker plots comparing the $\delta^{15}N$ between restored and unrestored sites by age category reveal and initial increase in $\delta^{15}N$ for the restored sites for the 0-2 age category followed by a general decreasing trend in $\delta^{15}N$ in restored sites as age category increases (Figure 3 and Table 3). The youngest restored floodplain sites (0-2 years old) have the highest concentrations of δ^{15}N with values reaching up to 10.78 (Figure 3 and Table 3). For the 0-2 and 2-5 age categories restored sites had higher δ^{15}N concentrations than unrestored sites and these observations support our hypothesis 2. Age categories 5-10 and 10-22 year old restorations had lower δ^{15}N concentrations than unrestored sites. The variability of δ^{15}N is the greatest in the 0-2 year age category supporting the hypothesis 3. The interquartile range is 7.41 compared to 3.075 for the 2-5 year age category, 2.28 for the 5-10 year age category, and 1.42 for the 10-22 year age category for restored sites (Figure 3 and Table 3).

Two sample t-tests showed no significant difference between restored and unrestored sites (Supplemental Figure 1) (F Ratio 0.4911 and Prob>F 0.4849). Paired t-tests confirmed no significant difference between restored and unrestored sites when analyzed as a whole but significant differences were discovered within pairs (Prob>F 0.0093) and among pairs (Prob>F 0.0373) (Supplemental Figure 2). Paired t-tests showed restored sites in the 0-2 and 2-5 age categories had significantly higher δ^{15}N than unrestored sites (Supplemental Figure 3). Unrestored sites in the 5-10 and 10-22 age categories had significantly higher δ^{15}N than restored sites (Supplemental Figure 3).

Temporal trajectory of 15N changes

- **H4**: Post restoration: Increased denitrification after restoration removes lighter ^{14}N via denitrification and increases δ^{15}N. But, as floodplains age (> 20 yrs), δ^{15}N decreases slightly as N availability decreases.

One way ANOVA analysis mirrors what was seen in Figure 5, δ^{15}N initially increases for the 0-2 age category for the restored sites and then decreases with site age. Restored sites in the 0-2 year age category had significantly higher δ^{15}N than the 10-22 year age category (p-Value 0.0063) (Supplemental Figure 4). Tukey-Kramer HSD connecting letters report mirrors this result with the 0-2 year age category categorized as A and 10-22 year age category as B

(Supplemental Figure 4). δ^{15}N for the restored 2-5 and 5-10 age categories were not significantly different from one another with Tukey-Kramer connecting letters of AB. The steady decrease in δ^{15}N can be seen in both the ANOVA graphic (Supplemental Figure 4) and a bivariate fit plot Figure 5). These results support hypothesis 4.

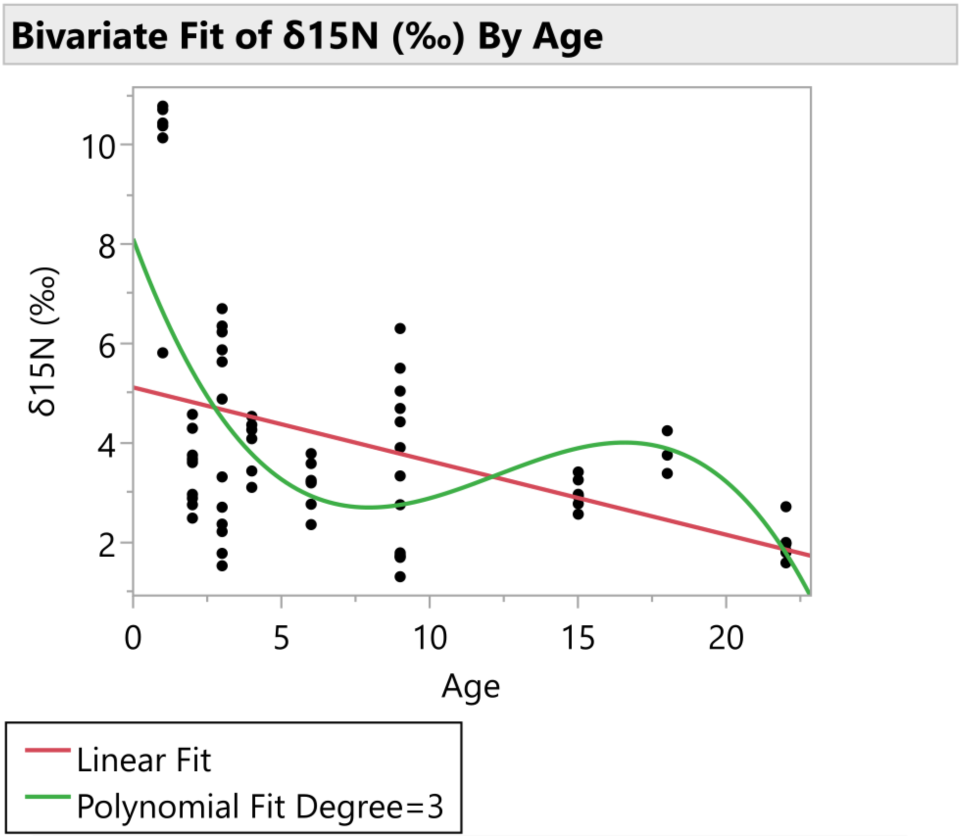
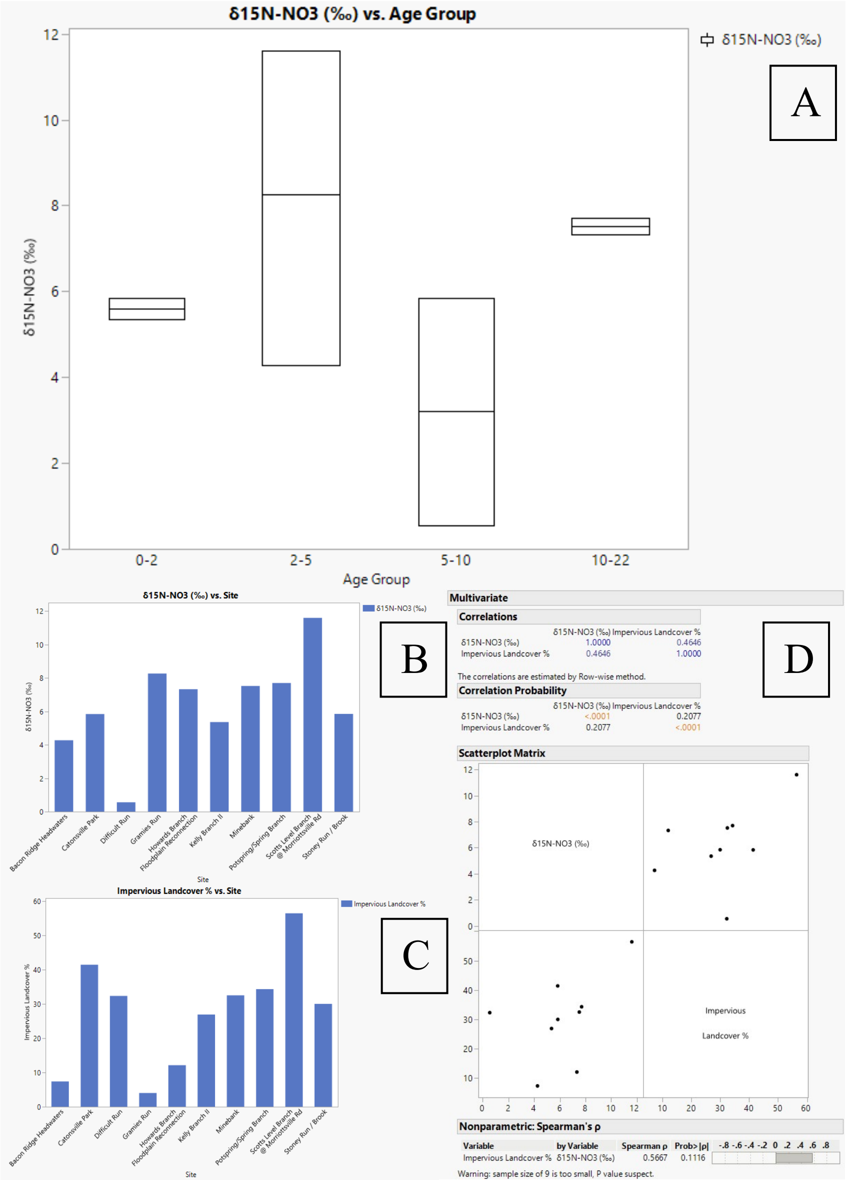


Figure 5: Bivariate plot of bulk soil δ^{15}N results at restored sites. δ^{15}N tended to decrease in concentration as site age category increased. 10-22 year old restorations had significantly lower δ^{15}N concentrations than 0-2 year old restorations (p-value 0.0063).

Changes in stream water nitrate isotopes for restoration sites by age category

Stream water δ^{15}N-NO_3^- concentrations did not have a significant trend over time when sites were grouped by age category (Figure 6 A). A positive trend was noted however when stream water δ^{15}N-NO_3^- was compared to a site's impervious surface cover (Figure 6 B and C). Pearson correlation between impervious surface cover and δ^{15}N-NO_3^- was 0.4646 (p 0.2077) and Spearman's nonparametric correlation was 0.5667 (p 0.1116) (Figure 6 D)



Correlations

	δ15N-NO3 (‰)	Impervious Landcover %
δ15N-NO3 (‰)	1.0000	0.4646
Impervious Landcover %	0.4646	1.0000

The correlations are estimated by Row-wise method.

Correlation Probability

	δ15N-NO3 (‰)	Impervious Landcover %
δ15N-NO3 (‰)	<.0001	0.2077
Impervious Landcover %	0.2077	<.0001

Nonparametric: Spearman's ρ

Variable	by Variable	Spearman ρ	Prob>|ρ|
Impervious Landcover %	δ15N-NO3 (‰)	0.5667	0.1116

Warning: sample size of 9 is too small, P value suspect.

Figure 6: Pearson and Spearman correlation coefficients between aqueous δ^{15}N and impervious surface cover.

Using stream water δ^{18}O-NO_3^- and δ^{15}N-NO_3^- values measured at the restoration sites a scatterplot was created (Figure 7). A framework developed by Kendall and McDonnel was then overlain on the datapoints. Using this framework, the relative abundance of δ^{18}O-NO_3^- and δ^{15}N-NO_3^- can then be used to determine the specific sources of aqueous nitrate (precipitation, fertilizer, sewage etc.) Our results indicated that only one site (Howards Branch) was likely to have nitrate sources from precipitation or possibly NO_3 fertilizer. The remainder of the sites were potentially affected by manure and septic waste, especially Scotts Level Branch which had the most elevated δ^{15}N values (11.6 ‰) and highest impervious surface coverage (54%). Difficult Run and Bacon Ridge fell within the overlap of NH_4 in fertilizer and rain and manure and septic waste and the remainder of the sites fell within the soil N profile. Broad Creek was excluded from the results as its values were below the detection threshold.

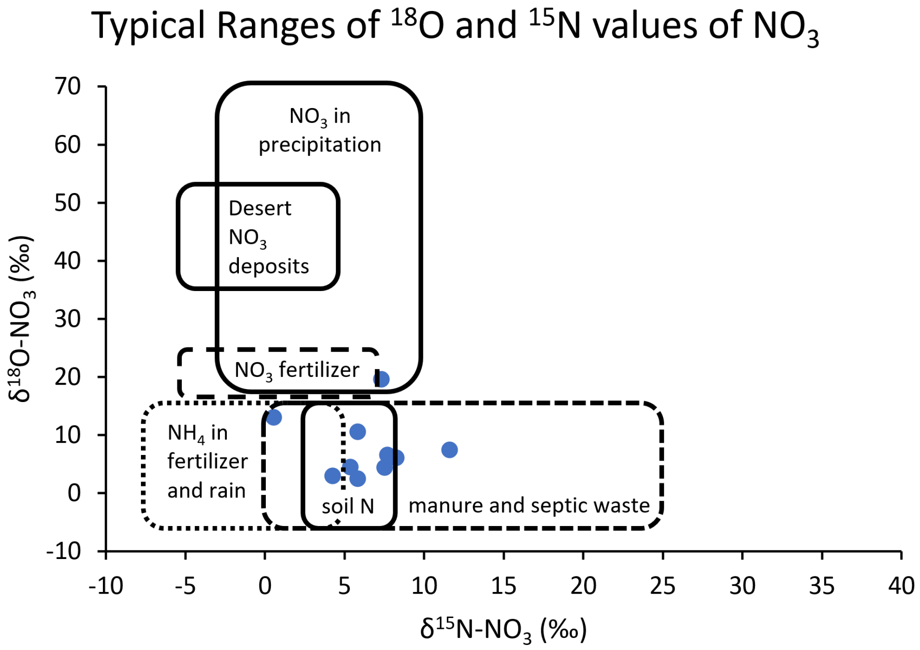


Figure 7: Illustration of the typical ranges of $\delta^{18}O\text{-}NO_3^-$ and $\delta^{15}N\text{-}NO_3^-$ values expected from various sources (adapted from Kendall and McDonnell, 1998).

Discussion

Changes in key floodplain soil properties with restoration

Although data are variable, unamended denitrification rate was found to increase with time (Figure 3A). Although there is high variability in the data as illustrated by the box and whisker plot, the 10-22 year age category had significantly higher denitrification at restored sites than unrestored sites. Other variables that significantly increased over time included % organic carbon, % organic matter, and soil moisture (Figure 3 B, C, E, Table 3). This indicates that the stream restorations are performing well over time, with likely improvements to ecosystem functions and measured improvements to denitrification. Significant increase in organic matter and organic carbon (Table 3) indicate that vegetative recovery after restoration is proceeding well and riparian plant growth is adding organic matter to the floodplain. Increased accumulation and processing of carbon can strongly regulate nitrogen dynamics in streams, and both riparian and wetland leaf litter is a major source of organic carbon and matter (Mayer *et al.*, 2022). As more organic material accumulates in the stream, more decomposition / denitrification occurs and in stream ecosystems it can be common for denitrification to be DOC limited (Groffman *et al.*, 2005; Taylor and Townsend, 2010).

In unrestored catchments which are prone to having higher peak flow and more “flashy” storm response characteristics, organic matter and organic carbon may be less likely to accumulate and remain trapped in interstitial zones or stay in debris jams and leaf packs, in turn diminishing the availability of organic matter in the floodplain soils (Groffman *et al.*, 2005). It is promising to see increasing DOC and organic matter concentrations at natural channel design restorations as it proves their efficacy. DOC is such a critical factor in microbial denitrification that restoration techniques are constantly being improved to supply greater quantities of DOC with more efficacy to floodplains and hyporheic zones in order to enhance denitrification and nitrogen removal (Duan *et al.*, 2019; Mayer *et al.*, 2022; Newcomer Johnson *et al.*, 2016).

In addition to increased organic matter and organic carbon being critical to denitrification more moist soil conditions are also necessary for bacterial metabolism (Tan *et al.*, 2018). The regrading and widened floodplain created during stream restoration increased soil moisture significantly (Table 3). Increased hyporheic exchange combined with the increased organic matter and organic carbon creates the ideal environment necessary for denitrification (Mayer *et al.*, 2022; Tan *et al.*, 2018).

Bulk density was found to have significantly decreased over time (Figure 3 D, Table 3) indicating recovery from compaction during restoration site construction and heavy machinery use (Laub *et al.*, 2013). This is a significant finding as constructed and restored wetlands often do not develop the full range of biogeochemical processes that normally form in natural wetland soils (Bruland *et al.*, 2006). Denitrification rates were affected as well in previous studies of restored wetlands, lending further credence that the restorations in this study were successful and did not degrade over time (Bruland *et al.*, 2006).

Particle size distribution remained constant among all age groups with the majority (50-70%) being sand, followed by silt (20-30%) and clay (10-20%) (Figure 4). Of note restored sites had smaller particle size distributions within categories, with % composition of sand, silt and clay being more consistent post-restoration. This is likely due to a combination of less “flashy” storm responses and a more homogonous sediment composition post-restoration (Lammers *et al.*, 2019). It is important that particle size distributions were not significantly altered as many aquatic species rely on a specific substrate for proper development and habitat (Sutherland *et al.*, 2010).

Changes in bulk soil $\delta^{15}N$ with restoration and its value for assessing restoration denitrification improvement

Results indicate that floodplain bulk soil $\delta^{15}N$ was not positively correlated with floodplain soil denitrification (whether it be amended and unamended) and did not increase with restoration age (Figure 3). Other than limited observations for 0-2 year age category our observations indicate that denitrification did not enrich the floodplain soil bulk soil $\delta^{15}N$. Given that $\delta^{15}N$ and denitrification do not co-vary (especially in the highest age category) it may be inadvisable to use $\delta^{15}N$ as a direct proxy for restoration efficacy or microbial denitrification. We hypothesize that the steady decrease in $\delta^{15}N$ observed over each consecutive time period could be due to increased vegetative growth which is often a major objective of stream restoration (Palmer *et al.*, 2014). With this increased vegetative biomass there is increasing organic N accumulation (with low $\delta^{15}N$) in the floodplain soil which depletes the $\delta^{15}N$ in the soil.

The composition of wetland and riparian species present at each site could also affect the $\delta^{15}N$ values recorded. Though not quantified in this study, N_2 fixing species like clover tend to introduce N into the soil through symbiotic fixation with a $\delta^{15}N$ at or near zero (Boeckx *et al.*, 2006; Irisarri *et al.*, 2019). Fertilizer use within each restoration's catchment may also impact $\delta^{15}N$ concentration with time. Organic fertilizers (often animal manure) contain elevated $\delta^{15}N$ values but mineral fertilizers (Haber-Bosch process ammonia) have a depleted signature (Boeckx *et al.*, 2006; Laura Vitòria, *et al.*, 2004).

In general a more "open" ecosystem with high denitrification and N losses tends to have more enriched $\delta^{15}N$ concentrations than more "closed" systems (Boeckx *et al.*, 2006; Michener and Lajtha, 2007). Openness here being defined as the relative importance of external N losses (mineralization, erosion and DON) to internal N cycling (Brenner *et al.*, 2001).

Stream water $\delta^{15}N\text{-}NO_3^-$ concentrations plotted by age group show no clear trend over time. The 2-5 and 5-10 age groups had the most variability. When broken down site by site the concentration of $\delta^{15}N\text{-}NO_3^-$ appeared to be influenced by land use and closely followed the impervious surface cover of the watershed ("StreamStats," 2022). The only site that did not follow this trend was Gramies Run which had ~ 50% agricultural (mostly pasture) surface cover but low impervious surface cover (4.03%). Gramies Run had 8.27 $\delta^{15}N\text{-}NO_3^-$ (‰), the second highest in this study so it was excluded from correlation analysis. The remaining sites showed a positive correlation with impervious surface cover. Pearsons's correlation between impervious surface cover and $\delta^{15}N\text{-}NO_3^-$ was 0.4646 (p 0.2077) and Spearman's nonparametric correlation was 0.5667 (p 0.1116) (Figure 6). While soil $\delta^{15}N$ was not a successful proxy for denitrification

Results indicated that only Howards Branch was likely to have nitrate sources from precipitation or possibly NO_3 fertilizer (Figure 7). The remainder of the sites were likely affected by manure and septic waste, especially Scotts Level Branch which had the most elevated $\delta^{15}N\text{-}NO_3^-$ values (11.6 ‰) and highest impervious surface coverage (54%) (Figure 7). We hypothesize that sewage contamination may have been present at Stoney Run as well as the site had elevated $\delta^{15}N\text{-}NO_3^-$ concentrations (5.85 ‰) and was being restored in part because of exposed and damaged sewage infrastructure. Difficult Run and Bacon Ridge fell within the overlap of NH_4 in fertilizer and rain and manure and septic waste and the remainder of the sites fell within the soil N profile (Figure 7). As most of the watersheds sampled had suburban, agricultural, and urban components to them it is likely that there was some degree of manure or septic waste contamination present at every site.

While we did collect soil samples from 12 sites with 10 samples from each site (six from restored and four from unrestored reaches), additional sampling would have benefited the study. The sampling was constrained from that originally proposed because of reduction in proposal budget (by nearly half that originally requested). This resulted in reduction of sample numbers by half. Future studies should increase the number of samples collected and the number of sites sampled for a more robust assessment of the changes in denitrification and bulk soil $\delta^{15}N$ values.

Conclusion

Though bulk soil $\delta^{15}N$ was not a promising candidate for monitoring denitrification in the floodplains of stream restorations other factors show promise that stream restorations in the Mid-Atlantic are accomplishing their planned goals. Denitrification, organic carbon, organic matter, and soil moisture all increased over time significantly at restored floodplains while bulk density significantly decreased over time. In concert these factors show that even after 22 years post restoration, restored streams are still evolving, and their flora and biomes are changing and evolving. Despite setbacks like soil compaction after restoration due to heavy machinery use, natural channel design is showing that it is a promising option for a variety of land uses and implementations. Particle size distribution of post restoration streams is not significantly influenced by the restoration but organic carbon accumulation suggests that peak stormflow is likely diminished and leaf litter and other organic detritus is allowed to accumulate.

Based on the results of this study we do not recommend the use of bulk soil $\delta^{15}N$ as a long term metric to assess denitrification effectiveness post restoration. Instead, other soil parameters such as soil organic matter, soil organic carbon, and moisture content could be used as proxy for denitrification. Our data, however, also shows that while these soil metrics could improve over time, the progress is slow and significant changes could take more than 10 years.

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Appendix 1: Selected photos of sampling sites (Restored and Unrestored sections)

Unrestored and Restored Howards Branch site

Unrestored and restored Scotts Level Branch at Marriottsville