FINAL REPORT:

USING STABLE NITROGEN ISOTOPES TO QUANTIFY THE INFLUENCE OF POTENTIAL NUTRIENT SOURCES IN THE PEACE RIVER WATERSHED

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Charlotte Harbor National Estuary Program
1926 Victoria Ave
Ft Myers, FL 33901

Prepared by:

Jason A. Hale and Renee M. Duffey

Charlotte Harbor Environmental Center
10941 Burnt Store Road
Punta Gorda, FL

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The Charlotte Harbor National Estuary Program is a partnership of citizens, elected officials, resource managers and commercial and recreational resource users working to improve the water quality and ecological integrity of the greater Charlotte Harbor watershed. A cooperative decision-making process is used within the program to address diverse resource management concerns in the 4,400 square mile study area. Many of these partners also financially support the Program, which, in turn, affords the Program opportunities to fund projects such as this. The entities that have financially supported the program include the following:

U.S. Environmental Protection Agency;
Southwest Florida Water Management District;
South Florida Water Management District;
Florida Department of Environmental Protection;
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Peace River/Manasota Region Water Supply Authority;
Polk, Sarasota, Manatee, Lee, Charlotte, DeSoto and Hardee Counties;
Cities of Sanibel, Cape Coral, Fort Myers, Punta Gorda, Venice, North Port and Fort Myers Beach;
and the Southwest Florida Regional Planning Council.

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EXECUTIVE SUMMARY

Due to high nutrient and phytoplankton abundance, water quality in some parts of Charlotte Harbor does not meet State of Florida standards for Class III surface waters. High concentrations of nutrients are associated with several water quality impairments in the Charlotte Harbor estuary. For example, chlorophyll concentration (as an index of phytoplankton) is related to the concentration of nutrients such as nitrogen and phosphorus. Phosphorus is abundant in upper Charlotte Harbor, as well as throughout the Peace and Myakka River watersheds, and is likely a result of high natural abundance as well as the product of particular land-uses. Nitrogen is often the focus of nutrient management and/or reduction strategies in Best Management Practices associated with land uses that occur in the Charlotte Harbor watershed, including those associated with the citrus industry, vegetable and agronomic crop production, and cow-calf operations. After identifying an impairment to water quality, understanding the quantitative relationships between surface water quality variables and basin land uses becomes a critical step towards restoring environmental integrity.

Estuaries like Charlotte Harbor are productive due to a natural supply of nutrients and freshwater from their watersheds. However, anthropogenic alteration of natural systems upstream can lead to cultural eutrophication of coastal areas, with generally negative results. When a water body's designated use, "maintenance of a healthy, well-balanced population of fish and wildlife" for example, is judged to be limited or impaired by a particular pollutant or condition, a "total maximum daily load" (TMDL) for the pollutant must be calculated. A TMDL is the maximum amount of a pollutant, such as nutrients or chlorophyll, that a water body can absorb and still maintain its designated use. Calculation of a TMDL requires the evaluation of the contributions of the pollutant from all basin land uses to the cumulative total load to a water body. Once a TMDL is calculated, activities in the watershed are compelled to assume some responsibility in reducing their contribution to the total amount of the pollutant in surface waters.

Initially, the goal of this project was to understand the link between surface water nutrients in Hardee and DeSoto counties with one watershed activity in particular: land application of residuals from domestic wastewater treatment plants (WWTP). Two-thirds of the residuals material produced in Florida are disposed of by land application. In 2002, 20% of the total amount of WWTP residuals applied across the state of Florida was delivered to sites in DeSoto County; and 30% of the total occurred within the Charlotte Harbor watershed, which accounts for only 7% of the total area of the state. In the following years, however, this practice was severely restricted in parts of DeSoto County. By the time this study began in 2007, land application of WWTP residuals in this area had dropped to a fraction of its former peak.

Our original hypotheses were based on the potential for substantial differences between WWTP residual stable nitrogen isotope ratio values ($\delta^{15}N$) and most other naturally occurring $\delta^{15}N$ values. This type of study involves the measurement of subtle differences in the distributions of stable isotopes across a range of compounds and conditions. All stable isotope analyses rely on biochemical processes (e.g. denitrification in a WWTP) to produce differences in the elemental signatures of target compounds. However, a wide range of potential $\delta^{15}N$ values also exists between "inorganic" (fertilizer based) and "organic" (livestock or human waste based) sources of nitrogen. Because inorganic and organic sources of nitrate are widespread across the study area, the reduction and elimination of land application sites for WWTP residuals across the study area did not require any change in methods or site selection, and made the distinction between "inorganic" and "organic" sources of nitrate an excellent alternative to our original project objective.

We hypothesized that patterns of presence, extent, and impact of nitrate from different sources on stream and river ecology in Hardee and DeSoto counties, Florida, could be recognized and described using stable nitrogen isotope analyses. The methods and results from this project will contribute to a variety of efforts intended to address issues raised by IWR impairments of surface waters within the Charlotte Harbor National Estuary Program study area.

We measured the stable nitrogen isotope ratio, $\delta^{15}N$ in nitrate, suspended particulate organic matter (SPOM, which includes phytoplankton), sediment organic matter, and stream-side vascular plants from twelve locations in Hardee and DeSoto counties, along the Peace River, Horse Creek, Joshua Creek, and Charlie Creek. Samples of each variable were collected at these sites during 8 sampling events between May and October, 2007, to characterize potential differences between dry and wet season conditions. To access existing ambient water quality monitoring data, many of our sampling locations were chosen based on proximity to Southwest Florida Water Management District - US Geological Survey, Peace River/ Manasota Regional Water Supply Authority, and Mosaic Company sampling programs.

One interesting result was the distribution of $\delta^{15}N$ in nitrate from “organic” and “inorganic” sources across the region, distributions that were unexpected when compared with published proportions of land use activities in each sub-basin. Using nitrate ambient concentration and $\delta^{15}N$ data, our mass balance equation estimated that 75% of the nitrate in upper Horse Creek and Charlie Creek may be supplied by inorganic sources, such as fertilizer from intensive agricultural land uses. Conversely, our data showed that approximately 60% of nitrate in Joshua Creek and lower Horse Creek (DeSoto County) is most likely from organic sources, such as livestock operations or compost. The $\delta^{15}N$ of nitrate along the length of the Peace River in both counties also consistently suggested organic sources of this nutrient.

Our analysis of the relationships between in surface water nitrate $\delta^{15}N$ and organic matter $\delta^{15}N$, either suspended in the water or settled on the bottom, suggested that some, but not all of the variation in organic matter $\delta^{15}N$ ratios comes from assimilation of nitrate, though other sources of nutrients, i.e. ammonia or dissolved organic matter, may provide nitrogen to both in-stream and down-stream production. A final result of our study was the wide ranges of $\delta^{15}N$ values for each variable at each location during our sampling period. Future studies measuring $\delta^{15}N$ in water quality variables should be aware that individual values may not represent the range of potential values at any location in this area.

These results should be helpful to efforts intending to calculate TMDL or nutrient thresholds for watershed activities in this area, as well as guide future projects concerned with tracing the impact of nutrients on the environment. Future projects should also consider the following: considered a wider range of sources, and sample them directly; consider the role of dissolved organic matter and in-stream nitrification in nitrogen cycle.

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INTRODUCTION

Background

Due to high nutrient and phytoplankton abundance, water quality in some parts of Charlotte Harbor does not meet State of Florida standards for Class III surface waters (Florida Department of Environmental Protection 2006). High concentrations of nutrients are associated with several water quality impairments in the Charlotte Harbor estuary. For example, chlorophyll concentration (as an index of phytoplankton) is related to the concentration of nutrients such as nitrogen and phosphorus. Phosphorus is abundant in upper Charlotte Harbor, as well as throughout the Peace and Myakka River watersheds, and is likely a combination of high natural abundance as well as the product of particular land-uses, (PBSJ 2007). Nitrogen is often the focus of nutrient management and/or reduction strategies in Best Management Practices (BMP) associated with land uses that occur in the Charlotte Harbor watershed, including the those associated with the citrus industry (Boman et al. 2004), vegetable and agronomic crop production (Bartnick et al. 2005), and cow-calf operations (Florida Cattlemen's Association 1999). After identifying water quality impairment, understanding the relationships between surface water quality variables and basin land uses becomes a critical step towards restoring water quality.

Estuaries like Charlotte Harbor are productive due to a natural supply of nutrients and freshwater from their watersheds. However, anthropogenic alteration of natural systems upstream can lead to cultural eutrophication of coastal areas, with generally negative results. When a water body's designated use, "maintenance of a healthy, well-balanced population of fish and wildlife" for example, is judged to be limited or impaired by a particular pollutant or condition, a "total maximum daily load" (TMDL) for the pollutant must be calculated. A TMDL is the maximum amount of a pollutant, such as nutrients or chlorophyll, that a water body can absorb and still maintain its designated use. Calculation of a TMDL requires the evaluation of the contributions of the pollutant from all basin land uses to the cumulative total load to a water body. Once a TMDL is calculated, activities in the watershed are compelled to assume some responsibility in reducing their contribution to the total amount of the pollutant in surface waters.

Ambient Water Quality Data

Once a water body has been judged impaired by excess nutrients, excess chlorophyll, or low dissolved oxygen, an analysis of the potential sources of those nutrients begins. *Total* nitrogen is the sum of all biologically available nitrogen in a system, and includes a number of different compounds: ammonia, nitrate and nitrite, urea, as well as other dissolved and particulate organic compounds that contain nitrogen. The compounds that bring a "tea stain", tannic, or colored appearance to river or lake water also contribute nitrogen compounds to *total* nitrogen. High values of total nitrogen in a river or lake may be a natural condition, or they may indicate a number of characteristics of unhealthy ecosystems – high concentrations of inorganic nutrients or dissolved organic matter, or excessive microbial and phytoplankton abundance.

Total nutrients and chlorophyll are the types of data used to detect impairments on water bodies. However, virtually all samples of surface water contain a mixture of nutrients from several sources, which may include fertilizer, decomposition of vegetable matter, effluent from wastewater treatment plant (WWTP), or particles brought by atmospheric deposition. Once nutrient streams from different land uses are mixed in a river or stream, it becomes impossible to discriminate which land use source of nutrients accounts for which fraction of nitrate using concentration data alone. Measuring the quality of water as it exits each parcel across a landscape is impractical, and when estimating non-point source nutrient contributions made by several sources in a watershed, it becomes difficult at best to estimate what percentages of the observed nitrate concentration are due to which sources. Industrial fertilizer dissolved in agricultural runoff, livestock operations, atmospheric deposition and land application of WWTP residuals may all contribute to the total pool of nutrients "upstream".

Some combinations of organic and inorganic nutrient information can be useful in discriminating nutrient sources. For instance, Total Kjeldahl Nitrogen (a composite of dissolved organic nitrogen and ammonia) may be used to identify “recent” inputs of nutrients from organic sources, especially related to livestock or other organic sources. Again, though, once nutrients from the different land uses are mixed in surface or ground water, it becomes extremely difficult to discriminate which sources account for which fractions using concentration data alone.

Stable Isotopes

Isotopes are forms of an element that differ slightly in atomic weight, but maintain the same biochemical functions (Peterson and Fry 1987). The only difference between two molecules that have incorporated different isotopes is that the molecule made with the “heavy” isotope weighs slightly more than the one made with the “light” isotope. For example, nitrogen has two stable (i.e. not radioactive) isotopes, denoted 14N and 15N. Either of these isotopes may be incorporated into the any compound containing nitrogen, e.g. nitrate, organic nitrogen compounds, or atmospheric nitrogen. Because any sample collected in nature contains a mixture of 14N and 15N, an isotopic ratio for the sample, R, is calculated by the following formula, which is the molecular ratio of heavy isotope to light isotope:

$$R = \frac{\text{(number of nitrogen molecules with atomic weight of 15)}}{\text{(number of nitrogen molecules with atomic weight of 14)}}$$

To compare isotope ratios of two samples, a second formula is used for each, comparing the R ratio of a sample to that of a universally accepted standard (the R for N_2 in air, for example), and represented by the “del” notation, δ:

$$\delta^{15}N = [(R_{sample}/R_{standard}) - 1] * 1000,$$

The value $\delta^{15}N$ is the one reported for each sample, in unit parts per thousand, ‰, pronounced and often written “permil”. For example, if a sample’s ratio of heavy to light isotope equals the “standard” ratio, the (R/R) quantity equals 1, and the sample’s $\delta^{15}N$ = 0‰ (it’s important to remember that a $\delta^{15}N$ value of 0‰ does not mean “zero” or “nothing”). Samples with more “heavy” nitrogen have increasingly positive values (greater than 0‰), while samples with less “heavy” nitrogen have negative values. When comparing two samples, the one with the higher ratio has a relatively greater proportion of the heavier isotope, and may be referred to as “enriched”, while the sample with the lighter ratio has relatively less heavy isotope, and is referred to as “depleted”.

Fractionation

Because the isotopically “lighter” molecules are transported at a slightly quicker rate than isotopically “heavier” molecules, processes such as nutrient assimilation, denitrification, and photosynthesis cause fractionation between the ratios of stable isotopes. Fractionation is a process that describes the cause of the difference in $\delta^{15}N$ ratios between a source (*e.g.* nutrients) and a product (*e.g.* phytoplankton), and there are many examples involving nitrogen compounds. Again, because a compound made with more 14N is lighter, phytoplankton assimilating ammonia will acquire the “lighter” ammonia molecules first. This process results in relatively “lighter” phytoplankton, while the remaining pool of ammonia becomes relatively “heavier” as ammonia molecules made with 15N becomes relatively more common (Peterson and Fry 1987). In other words, the process of nutrient assimilation initially produces biomass (*i.e.* phytoplankton) with a $\delta^{15}N$ related to, but lighter than, its nutrient source. However, because the remaining ammonia becomes relatively “heavier”, phytoplankton become progressively “heavier” as well as they assimilate more of the remaining ammonia composed of 15N. Similarly, plants, microbes, and bacteria all fractionate between isotopes when assimilating nitrate into biomass; “lighter” nitrate is assimilated, leaving behind a “heavier” pool. As a rule, the *remaining* source material will become enriched (heavier), while the *products* of the reaction or processes become relatively depleted (lighter).

Because of this relationship between source and product, stable isotope ratios can be useful for determining potential sources of nutrients. For example, consider excess nutrients from two different sources – vegetable crops and processed wastewater – carried by runoff into the same stream. Inorganic nitrogen from “inorganic” artificial fertilizer bears a $\delta^{15}N$ value characteristic of the industrial processes that produced it, which is generally less than 0‰ (Peterson and Fry 1987). For example, Curt et al. (2004) recorded a $\delta^{15}N$ of -1.59 ‰ in granular fertilizer, and Hale et al. (In Review) recorded a $\delta^{15}N$ of -8.9 ‰ for ammonia from dissolved fertilizer. On the other hand, repeated denitrification and other microbial processes occurring during wastewater treatment generally produce both nitrate and organic nitrogen with enriched ratios greater than +10‰ (review in Valiela et al. 2000). Other “organic” sources of nitrogen include beef and dairy cattle operations, which may produce $\delta^{15}N$ of +14 ‰ (Curt et al. 2004). Treatment facilities that employ an alternating aerobic/anaerobic cycle to achieve a higher degree of nutrient reduction may generate residuals which have $\delta^{15}N$ in nitrate substantially more enriched than most materials found in nature, as high as +26‰ (Savage and Elmgren 2004), +30‰ (Hale et al., In Review) and +40‰ (Jordan et al. 1997).

In this example, water bearing nutrients with light ratios, near 0 ‰, becomes mixed with water containing nutrients with heavier ratios, perhaps +20 ‰ or more, and the resultant $\delta^{15}N$ of the nutrient downstream will be some value between 0 and +20 ‰. Given these potential maximum and minimum values, and the total concentration of the nutrient, the contribution of each land use can be calculated. To complete the example, if the resulting value is +5 ‰, 75% of the nitrate in the stream originated from the vegetable crops operation, while 25% originated from the WWTP effluent. However, an ambient water quality monitoring program measuring only the concentration of nutrients downstream will not be able to identify either the sources of nutrient or their relative contributions the total amount. It follows that, when combined with conventional nutrient data, the potential for $\delta^{15}N$ analyses to identify nutrient sources and processes may present a broader view of watershed processes, and may be particularly useful to direct resource management activities toward the most efficient actions. These may include contribution to calculations of TMDL, or evaluation of BMPs intended to reduce nutrient in runoff.

Original Project Objective

Initially, the goal of this project was to understand the link between surface water nutrients in Hardee and DeSoto counties (and ultimately Charlotte Harbor) and one watershed activity in particular: land application of residuals from domestic wastewater treatment plants (WWTP). Two thirds of Florida’s citizens are served by centralized sewage treatment plants, and many of these plants are located in southwest Florida (Florida DEP 2007). Though technologies differ with plant age and design, the residual materials produced by these plants still require management after the wastewater treatment process. Management practices include burying in landfills, injection into deep aquifers, surface release into treatment wetlands, or application to lands where it may be considered fertilizer.

Two-thirds of the domestic WWTP residual material produced in Florida are disposed of by land application. In 2002, 20% of the total amount of WWTP residuals applied across the state of Florida was delivered to sites in DeSoto County; and 30% of the total occurred within the Charlotte Harbor watershed (Florida DEP 2007), which accounts for only 7% of the total area of the state. To contribute to the understanding of the role of this land use activity in the delivery of nutrients to surface waters in the Charlotte Harbor National Estuary Program study area, the original goal of this project was to trace nutrients from land application sites using $\delta^{15}N$ analyses. In the years after 2002, however, this practice was severely restricted in parts of DeSoto County. By the time this study began in 2007, the area of land used for the disposal of WWTP residuals had dropped to a fraction of its former peak (Florida DEP 2007).

As described above, $\delta^{15}N$ analyses rely on fractionation to produce different $\delta^{15}N$ values in environmental samples. Our original hypotheses were based on potential for substantial differences between WWTP residual $\delta^{15}N$ and most other naturally occurring $\delta^{15}N$ ratios noted above. However, also as described above, a wide range of potential $\delta^{15}N$ values exists between “inorganic” and “organic” sources of nitrogen, making this distinction between sources an excellent alternative to our original project objective. Artificial fertilizer is an “inorganic” source of nitrate, while “organic” sources include beef, dairy, or other livestock operations, compost, and human waste, either from septage or land application of WWTP residuals.

Because these examples of organic and inorganic sources of nitrate are widespread across the study area, the reduction and elimination of land application sites for WWTP residuals across the study area did not require any change in our project goals, hypotheses, methods, or in the selection of our sampling locations.

OBJECTIVES

We hypothesized that patterns of the presence, extent, and impact of nitrate from particular land uses on surface water quality in the Peace River basin could be recognized and described using stable nitrogen isotope analyses. All stable isotope analyses rely on environmental processes to produce differences in the elemental signatures of target compounds, and our hypotheses are based on the differences in stable nitrogen isotope ratios between “inorganic” sources of nitrate, such as artificial fertilizer, and “organic” sources, such as manure, and compost and WWTP residuals.

Our objectives were first, to trace those contributions to three environmental response variables, namely suspended particulate organic matter in surface waters, benthic organic matter, and stream-side vegetation; second, to quantify the relative contributions to total nitrate in surface waters from these two broad types of sources, “inorganic” and “organic”. We expect the results from this project will contribute to the evaluation of efforts focused on reducing total nitrogen in the Peace River and its tributaries, as well as the total amount of nutrients reaching the Charlotte Harbor estuary.

METHODS

Sampling Sites and Ambient Water Quality Data

Sampling locations were located in Hardee, DeSoto, and Charlotte counties (Fig. 1). The Peace River Cumulative Impact Study (PBSJ 2007) characterizes land uses in Hardee and DeSoto counties as “developed for intensive agriculture or range, though native upland habitats and wetlands are conserved in some areas”. While mining has not played a direct role in land conversion in this area at the time of this project, it has been cited as contributing to major water quality issues in the Peace River (PBSJ 2007). Thus, excess nutrients from these activities may include (a) artificial fertilizer, (b) organic sources, compost, manure, WWTP residuals, and others, or (c) both.

Within Hardee and DeSoto Counties, 12 sites were chosen for ease of access as well as availability of complementary water quality and flow data: Joshua Creek (2 sites), Charlie Creek (2 sites), and Horse Creek (4 sites), and the Peace River (4 sites) (of these, only one was located in Charlotte County). One additional requirement – that water flow be visible – was used to determine the location of upstream stations for the 3 creek sub-basins. During initial site evaluations, flow was not detected at Charlie Creek at SR64, Joshua Creek at SR70, or Horse Creek at SR62; these observations restricted the total stream length that could be sampled during this project.

To capture potential differences in environmental conditions between wet and dry seasons, all sampling took place from May through October, 2007. The wet season in this region is generally characterized as occurring from June through October (e.g. PBSJ 2007). However, to verify this distinction and guide statistical analyses based on season and station, stream flow data recorded at locations on the three creeks and two locations along the Peace River coincident or near to our sampling stations were acquired from the USGS Instantaneous Data Archive (http://ida.water.usgs.gov/ida/). Water quality data from monthly monitoring programs was obtained from Peace River/ Manasota Regional Water Supply Authority (PRMRWSA) and Mosaic Co. (Horse Creek stations); PRMRWSA (station PR01); USGS and Southwest Florida Water Management District (all other stations).

Sample Collection

Sites were visited during 8 sampling events, and most sampling events took 2 field days to complete: May 16, 18; June 9, 10; July 25, 27; August 2, 3; August 22, 24; September 19; October 4; and October 10.

Four types of environmental variables were collected for stable nitrogen isotope analyses: filtered water (for nitrate), suspended particulate organic matter (SPOM); benthic organic matter, and leaves from a stream-side tree.

Sample Preparation and Processing Environmental Samples for $\delta^{15}N$

Nitrate. Whole water was filtered through pre-combusted quartz fiber filters (nominal pore size < 10 µm) for use in the natural abundance $\delta^{15}N$ in nitrate procedure following Sigman et al. (1997). Briefly, duplicate flasks of 400 ml of filtered water were treated with sodium chloride (for osmotic balance) and buffered with magnesium oxide to a pH of approximately 9.6, and then incubated with lids off at 65 °C. This step converted all reduced inorganic nitrogen (ammonia and ammonium ion) to the volatile ammonia form, which evaporated. Samples were incubated to dryness, and then rehydrated just prior to the ammonium diffusion step. Devarda's alloy was added to reduce the remaining inorganic nitrogen compounds (nitrate and nitrite) to ammonia, and an enclosed Teflon packet containing an acidified disk (a drop of concentrated sulfuric acid on a 1-cm GF/D filter) was immediately added to each flask, which was then tightly capped. Next, samples were incubated at 65 °C for 4 days, and then gently shaken for 4 days allowing ammonia to concentrate in the flask headspace and be "captured" on the acidified disk. After this time, packets were removed from sample flasks, partially opened, and dried at 65 °C, and stored in a desiccator until the day of analysis. Duplicate sub-samples were combined for each station at each sampling event. Acidified disks were analyzed for $\delta^{15}N$.

Organic Matter. To determine the fate of nitrate in surface waters, two types of in-stream organic matter were collected. At each site visit, up to four liters of whole water were passed though quartz fiber filters (for the $\delta^{15}N$ in nitrate) procedure; these filters were then dried at 65 °C. Once dried, the thin layer of organic material was peeled off, loaded directly into sample boats, and analyzed in duplicate for $\delta^{15}N$.

Organic matter on the stream botton was sampled by skimming the top 0.5 cm of floc, benthic microalgae, and sediment into a clean 50-ml centrifuge vial. Samples were allowed to settle, decanted of excess water, and frozen until preparation time. Samples were thawed and dried in the vials at 65 °C, and organic matter was then collected as a top layer of dried crust into clean glass scintillation vials, and stored in a desiccator. Before processing, samples were loaded into boats, and analyzed in duplicate for $\delta^{15}N$.

Stream-side Vegetation. To determine the relationship between nitrate in surface waters and plants in or adjacent to streams, the same individual Carolina willow trees (S*alix caroliniana)* were sampled at each station during each sampling event except for the Peace River estuary station, PR01, where no willow trees were available – instead, a red mangrove, *Rhizophora mangle*, was chosen. Both of these species tolerate hydric soils, and were chosen based on being the most likely to contact soils flooded with river water, and thus be exposed to nitrate. In all cases, young leaves from the same tree at each site were collected at each sampling event, dried at 65 °C, ground by mortar and pestle, and analyzed in duplicate for $\delta^{15}N$.

All $\delta^{15}N$ samples were analyzed at the Stable Isotope Lab at the Dept. of Geological Sciences, UF in October and November, 2007, on a Carlos Erba CNS Analyzer. Internal precision of 0.02‰ was evaluated on each run.

Data Analysis

An ANOVA was used to compare mean $\delta^{15}N$ in nitrate between dry and wet seasons. To describe relationships between $\delta^{15}N$ in nitrate and $\delta^{15}N$ in organic matter associated with streams, two-way ANOVAs were conducted to detect effects due to station location along stream length (upstream vs. downstream), within creek basins (Joshua, Charlie, Horse creeks), and an undefined characteristic related to stream size, i.e. non-linear change of wetted surface area with flow volume (creeks vs. Peace River). A two-sample t-test was used to detect a difference between the in-stream organic matter samples, sediment and suspended particulate organic matter. To illustrate regional trends in nitrate concentration and source, concentration (mg/L) data from ambient monthly monitoring programs and our observed δ^{15}N in nitrate were plotted on an area map depicting sampling locations, streams, and sub-basin boundaries.

All statistical analyses were made using S-plus 2000 (Mathsoft 1999). As sample sizes were frequently uneven in ANOVA tests (see Tab. 2), unbalanced Type III tests were used (Mathsoft 1999). Treatment effects tested by ANOVA were judged significant at high F-ratio values and $p < 0.05$.

Mass Balance

A literature survey was used to complete a table of expected values for potential land-uses in the study area. Curt et al. (2004) provided numerous examples, including various livestock, human, and fertilizer. Using concentration of nitrate in streams based on ambient water quality monitoring data described above, our observed δ15N in nitrate data, and these literature values, we used a simple, 2-component mass balance formula (Phillips 2001; Phillips and Gregg 2003) to estimate contributions of broad land-uses to total nitrate concentration based on δ^{15}N in nitrate by:

$$\delta^{15}\mathrm{N(obs)} = f(A)^{*}\,\delta^{15}\mathrm{N}(A) + f(B)^{*}\,\delta^{15}\mathrm{N}(B),$$

and

$$1 = f(A) + f(B),$$

where δ^{15}N(obs) represents the mean δ^{15}N in nitrate observed at a station; δ^{15}N(A) and δ^{15}N(B) are δ^{15}N in nitrate associated with two land uses, A and B, which were based on literature values; and f(A) and f(B) are fractions of observed nitrate concentration.

In most cases, “groundwater contaminated with inorganic fertilizer” was used as the most depleted nutrient source component because the estimate of Curt et al. (2004) was most precisely documented (1.48 ‰). However, because the mean δ^{15}N in the upper Horse Creek sub-basin was so low, the generally cited value of -1‰ (Peterson and Fry 1987) was substituted for this location. To represent the enriched nutrient source component, land use practices in the sub-basin descriptions of PBSJ (2007) were associated with available estimates from the literature survey, including data from Curt et al. (2004) and the review in McClelland and Valiela (1998).

RESULTS

Sampling Sites and Preliminary Analyses

Table 1 includes the number of samples of each variable analyzed for each station, reflecting a maximum of 8 sampling events (missing samples were due to either restricted access to site or lab error). The distinction between dry and wet seasons was not apparent until after July 1, 2007 (Fig. 2). Therefore, to test for a seasonal difference in δ^{15}N in nitrate, we used our sampling events before this date to characterize dry season conditions, and samples collected after this date to characterize wet season conditions. We found no difference in mean δ^{15}N in nitrate values between dry and wet seasons (ANOVA: F(1, 85) = 1.809, p=0.18). Therefore, all measurements of δ^{15}N in nitrate at each station were pooled for all subsequent analyses.

δ^{15}N in Environmental Samples

δ^{15}N in Nitrate. The highest (most enriched) observed values of δ^{15}N in nitrate occurred at the lower Horse Creek stations, HC72 and HC769, 13.44‰ and 12.42‰, respectively (Fig. 3). High maximum values also occurred at both Joshua Creek stations, JC31 and JC17. Conversely, the lowest observations of δ^{15}N in nitrate were made at the upper Charlie Creek station, -1.98‰ at CC66, and the upper Horse Creek stations, HC64 and HCL, -3.04‰ and -1.49‰ , respectively. The ranges of values observed at some stations were wide, as well, often spanning 6‰ or more. Another noticeable pattern was that downstream stations generally increased in both mean and range of values, though this was not always the case. The highest mean $\delta^{15}N$ in nitrate was at Joshua Creek at SR17, while the range at that station was among the most narrow. Other stations with narrow ranges included Charlie Creek at SR66, Horse Creek at SR 64, and Peace River at CR760 (ranges ~ 4‰, 3‰, and 2‰, respectively).

One other observation is important to point out. The highest single observation of $\delta^{15}N$ in nitrate for the Peace River occurs as the river enters our study area from Polk County, at station PRW; maximum values decreases downstream from here. However, conditions change by Gardner (station PRG), near the Hardee/DeSoto county line, just downstream from the confluence with Charlie Creek, where lower maximum and mean $\delta^{15}N$ in nitrate were observed.

$\delta^{15}N$ in Organic Matter. $\delta^{15}N$ values in SPOM and sediment organic matter measured at the each station were significantly different (paired two-sample t-test, df = 71, $p < 0.05$), suggesting the influence of different nutrient sources, different microbial/ phytoplankton communities, or both. Downstream samples of both SPOM and sediment organic were generally more enriched than upstream samples (Fig. 4).

The relationship between $\delta^{15}N$ in nitrate and sediment organic matter ratio was affected by sub-basin-specific characteristics (2-way ANOVA: F(3,68) = 4.502, $p < 0.05$), indicating significant basin effects. However, the relationships between $\delta^{15}N$ in nitrate and $\delta^{15}N$ of both sediment organic matter (2-way ANOVA: F(1,76) = 17.820, $p < 0.05$) and suspended particulate organic matter (SPOM) (2-way ANOVA: F(1,68) = 6.219, $p < 0.05$) were also affected by some difference in stream size, i.e. creek or river.

$\delta^{15}N$ in Vegetation. Although the $\delta^{15}N$ of young *Salix* and *Rhizophora* leaves were dependent on station (2-way ANOVA, main effect F(11, 78) = 3.260, $p < 0.05$), they were not related to $\delta^{15}N$ in nitrate (2-way ANOVA, main effect F(1, 88) = 0.302, p = 0.863) , and no interaction between $\delta^{15}N$ in nitrate and station was detected (2-way ANOVA: F(11,57) = 0.861, p = 0.582). Indeed, very little of the variability in new leaf $\delta^{15}N$ was explained by in-stream $\delta^{15}N$ in nitrate (slope = 0.24, $p < 0.05$, $r^2 = 0.19$ n.s.). The upstream-downstream patterns visible in $\delta^{15}N$ of SPOM and sediment organic matter were not apparent in stream-side vegetation $\delta^{15}N$ (Fig. 4).

Map of Nitrate Concentration and $\delta^{15}N$

A map of the distribution of nitrate concentration and stable nitrogen isotope ratio illustrates a pair of contrasting observations (Fig. 5). First, there is a pattern of enrichment from upstream to downstream locations in Charlie, Joshua, and Horse creeks. This is also evident in Figure 3. However, it is particularly clear how both $\delta^{15}N$ in nitrate and nitrate concentration increase along Horse Creek, from the upstream stations in Hardee County to the downstream stations in DeSoto County. Second, as noted in Figure 3, there seems to be a general decrease in $\delta^{15}N$ in nitrate from upstream to downstream along the Peace River.

Mass Balance

A wide range of values characterized mean ($n \leq 8$) $\delta^{15}N$ in nitrate in sub-basin creeks, from near 0‰ to almost 10‰. Similarly, our estimated mean summer concentrations of nitrate in these creeks spanned ranged from 0.16 to 0.52 mg/L. According to our mass balance calculations, the lowest mean nitrate concentrations occurred in Charlie Creek and upper Horse Creek, where inorganic fertilizer may account for approximately 3/4 of the nitrate in those streams (Tab. 3). In contrast, after passing through the northwest portion of DeSoto County, the mean nitrate concentration in the lower half of Horse Creek doubles, and organic sources of nitrate seem to be responsible. In this part of the creek, we calculate that the source of just under half of this nitrate may be inorganic fertilizer, while greater than half comes from an organic source, such as livestock.

DISCUSSION

Variability in δ^{15}N Values

At most locations, δ^{15}N in nitrate values varied greatly, indicating that the *proportion* of total nitrate from potential sources may vary even as concentration - and the amount of nitrate delivered downstream - changes. This suggests that, as with ambient water quality monitoring, several observations of δ^{15}N should be made to adequately characterize conditions at any single location – under-sampling could lead to erroneous conclusions.

Our results do show significant site effects, suggesting that sub-basins – or portions of sub-basins in the case of Horse Creek – exhibit characteristic δ^{15}N values that can be discriminated from each other. These characteristic δ^{15}N in nitrate values may be a much more quantitative way to relate the impact a particular land use or activity has on surface water in the context of excess nutrients and the IWR than by comparing total area or proportion of basin area dedicated.

Mean δ^{15}N in nitrate across sub-basins fell within a wide range (Fig. 2 and Table 3), varying from a depleted 2.10‰ in Charlie Creek to an enriched 8.36‰ for Joshua Creek. And although within-station variability was often high, 6‰ provides a consistent breakpoint among groups of stations. Stations with a mean δ^{15}N in nitrate value below 6‰ include both Charlie Creek stations and the two stations characterizing upper Horse Creek. Conversely, mean δ^{15}N values at the two lower Horse Creek and both Joshua Creek stations locations were greater than 6‰. As land uses are comparatively similar between the Charlie and Joshua Creek sub-basins (i.e. “intensive agriculture”; PBSJ 2007), this is an interesting difference, and implies the presence of a considerably enriched source of nitrate in the Joshua Creek sub-basin. This source could include septage or animal manure spread as fertilizer, or any other land use involving livestock. Conversely, the consistently lower mean δ^{15}N in nitrate values observed in Charlie Creek and some upper Horse Creek stations suggest inorganic sources of fertilizer. The four Peace River stations were variable along the length of the river, and did not exhibit a similar pattern.

The enriched ratios observed in Joshua Creek are probably not related to the of application of WWTP residuals, as the last LAS in the Joshua Creek sub-basin closed sometime before the start of sampling for this project (C. Minskey, pers. comm.). Similarly, use of land application sites for disposal of domestic WWTP residuals in the lower Horse Creek sub-basin has been particularly restricted since 2003, so this result implies inputs of enriched nitrate from other sources, such as livestock operations. It should be noted that as of late 2007, a permit for an industrial residuals spreading operation had been submitted to FDEP (C. Minskey pers. comm.). If approved, it would be difficult to predict the influence of this activity on the isotope ratios observed in lower Horse Creek without knowing the content of the materials to be applied. Land application sites in other counties that accept industrial residuals are supplied by citrus companies, among other industries, and data on the distribution of δ^{15}N in this type of material, as well as its intended use (e.g. livestock feed) would be required, though not difficult to collect.

Variability in Relationship between Nitrate and Organic Matter: δ^{15}N

It is interesting to note that we did not find a significant interaction from the sub-basin treatment on the relationship between δ^{15}N in nitrate and δ^{15}N of SPOM. One possible reason for this is that SPOM may be using a nitrogen source other than nitrate, i.e. ammonium ion. Higher ammonium ion concentration may promote variable or lower δ^{15}N in nitrate due to its (indirect) effect on nitrate uptake rate (Queguiner et al. 1986), and thus cause less uptake and subsequent fractionation of remaining δ^{15}N in nitrate. This hypothesis could be easily tested by sampling δ^{15}N in ammonia. Alternatively, phytoplankton and water column microbes may not be using dissolved inorganic nitrogen at all, but consuming and assimilating dissolved organic matter and converting it into necessary nitrogen compounds as found in coastal phytoplankton communities by Carlsson et al. (1995).

There are several factors which may influence the relationship between SPOM and nitrate. Fractionation – the difference between the δ^{15}N of a nutrient source and the δ^{15}N of phytoplankton, or microbes, or plants that assimilate it – can itself be affected by a number of factors, including nutrient abundance, cell growth rate, and cell uptake rate. Nonetheless, these associations between nitrate and organic matter suggest that $\delta^{15}N$ of organic matter can be used as a broad indicator of nitrate source, saving the considerable expense involved in $\delta^{15}N$ sampling of water column inorganic nitrogen. However, because of the relatively poor model fit, further sampling of δ15N in both ammonia and nitrate is recommended.

Trees along the stream bank were not dependent on nitrogen from streams for growth during the project period, and were more influenced by site-specific effects, which may include site sediment characteristics, tree age, or some other site-specific stress. Emergent or submerged vegetation might provide a different result because the roots of those plants are established within soils permanently flooded by river water, and can be expected to respond more consistently to stream conditions (Cole 2004).

Mass Balance, and Geographic Patterns of $\delta^{15}N$ in Nitrate

The mean concentration of nitrate in Joshua Creek was the highest of the sub-basins in our study area, and the nitrogen isotope ratio was often between 9‰ and 10‰. This in spite of a predominant sub-basin land use described as intense agriculture (PBSJ 2007), including a high proportion of citrus grove, which would imply a lighter isotope signal if inorganic fertilizer use was widespread. An alternative explanation may be that citrus growers in the Joshua Creek sub-basin fertilize with animal manure or septage. Nonetheless, assuming an isotope ratio of 14‰, an organic source of nutrients may be responsible for 2/3 of the nitrate in Joshua Creek, while inorganic fertilizer may be supply the remaining 1/3. Fogg et al. (1998) provides an important review of the effects of soils on observed isotope ratios attributed to a variety of land uses, an additional consideration among some of the sub-basins sampled in this study. This result does raise an important question regarding the conception of particular land uses and the amount of watershed area they occupy.

The highest single observation of $\delta^{15}N$ in nitrate in our study area occurred in Horse Creek, in DeSoto County, and both Horse Creek stations in DeSoto County show substantially higher nitrate concentration and more enriched $\delta^{15}N$ in nitrate than other stations. It is unknown whether this is a remnant of past land use practices, i.e. continued leaching of nitrate originating from residuals spreading occurring up to 2002, or current land use activities. Permission to access current or historic land application sites on private property is difficult to obtain (Hale, unpubl. data). However, these markedly enriched values occurring in DeSoto County locations – lower Horse Creek and Joshua Creek – present evidence to support future monitoring of all nutrient sources in this area.

The highest nitrate concentration observed in our study area occurs at our first sampling location along the Peace River in Hardee County. In general, $\delta^{15}N$ in nitrate in the Peace River decreases from upstream to downstream, which is in contrast to the three streams in our study. Polk County lies upstream from all Peace River stations, and includes the headwaters of the Peace River. In addition to a variety of intensive land uses, including mining, Polk County receives more WWTP residuals for spreading than any other single county in the state, over 60% more than the next highest county (Florida DEP 2007). However, if a large proportion of the nitrate measured at PRW originated from WWTP residuals in Polk County, the $\delta^{15}N$ in nitrate would likely be higher, closer in range with the maximum values in Joshua Creek and the DeSoto County Horse Creek stations. A $\delta^{15}N$ study of nutrient sources contributing to Peace River nutrients seems warranted.

CONCLUSIONS

The methods used and results produced by this project have many implications for future research and monitoring programs that may support issues associated with the IWR. Besides those mentioned above, additional research and monitoring efforts that would support TMDL calculation and BMP evaluation include:

- Aquatic food web analyses. Because suspended particulate organic matter and benthic organic matter are food for stream invertebrates and fishes (e.g. Hollander and Peebles 2004), stable isotope analysis of these primary and secondary consumers can contribute to a food web analysis to evaluate stream health or stream restoration efforts that may be mandated by IWR;

- Other stable isotopes (i.e.$\delta^{18}O$, $\delta^{13}C$) may be analyzed in these and other compounds to trace sources of inorganic phosphorus, discriminate between organic sources of nitrate, and define the role of dissolved organic matter in riverine and estuarine production, especially in questions of estuarine hypoxia.
- The contribution of dissolved organic matter processing by microbes to observed nitrate and ammonia concentrations; and
- The contribution of atmospheric deposition to a basin-wide total nitrogen budget.

Application of some of the methods in this project to actual TMDL calculations would require several more steps. First, discrete samples of all potential sources should be analyzed. This project used literature values to provide estimates of $\delta^{15}N$ from different activities; actual estimates of $\delta^{15}N$ from livestock operations, intensive row crops, mining effluent, and atmospheric deposition would be more appropriate. Second, we used a simple, 2-component mixing model -- a more sophisticated model would be required to combine values from more than two sources e.g. Phillips and Gregg (2003), Voss et al. (2006). However, this project will still serve as a valuable first step in planning future sampling efforts, as well as comparing future results.

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SOURCE OF UNPUBLISHED DATA

Minskey, C. Residuals Specialist, Domestic Wastewater Section, Florida DEP. 2600 Blair Stone Road. Tallahassee, FL.

TABLES AND FIGURES

Table 1. $\delta^{15}N$ sample size for each station and variable (maximum 8 sampling events).

Location	Station name	NO3	SPOM	Sediment OM	Plant
Peace River near river mouth	PR01	7	6	7	8
Peace River near Nocatee	PR760	7	5	6	7
Peace River at Gardner	PRG	8	6	7	8
Peace River in Wauchula	PRW	7	6	7	7
Horse Creek at CR 769	HC769	7	7	8	7
Horse Creek at SR 72	HC72	8	8	8	8
Horse Creek near Lily Community	HCL	7	5	6	7
Horse Creek at SR64	HC64	8	7	8	7
Charlie Creek at US17	CC17	7	8	8	8
Charlie Creek at SR66	CC66	7	7	7	8
Joshua Creek at US17	JC17	7	7	7	7
Joshua Creek at SR31	JC31	7	7	7	7

Table 2a. Mean and range of δ^{15}N in nitrate samples by sub-basin.

	mean δN15	minimum, maximum
Study Area (12 stations)	4.81 ‰	-3.04 ‰, 13.44 ‰
Peace River (4 stations)	5.69	-1.01, 9.18
Horse Creek (4 stations)	3.55	-3.04, 13.44
Charlie Creek (2 stations)	2.10	-1.98, 9.57
Joshua Creek (2 stations)	8.36	1.84, 11.59

Table 2b. Mean and range of δ^{15}N of suspended particulate organic matter samples, by sub-basin.

	mean δN15	minimum, maximum
Study Area (12 stations)	4.24	1.02, 5.99
Peace River (4 stations)	4.92	5.99, 8.00
Horse Creek (4 stations)	3.63	7.00, 8.00
Charlie Creek (2 stations)	2.34	1.41, 3.13
Joshua Creek (2 stations)	5.34	4.93, 5.92

Table2c. Mean and range of δ^{15}N of stream sediment organic matter samples, by sub-basin.

	mean δN15	minimum, maximum
Study Area (12 stations)	4.55	1.21, 8.07
Peace River (4 stations)	5.23	3.05, 8.07
Horse Creek (4 stations)	3.87	1.21, 7.94
Charlie Creek (2 stations)	3.55	2.25, 5.73
Joshua Creek (2 stations)	5.63	3.89, 7.12

Table 2d. Mean and range of δ^{15}N of young vascular leaf samples, by sub-basin.

	mean δN15	minimum, maximum
Study Area (12 stations)	5.00	0.78, 11.31
Peace River (4 stations)	7.00	4.17, 11.31
Horse Creek (4 stations)	3.47	0.78, 6.78
Charlie Creek (2 stations)	4.48	2.13, 6.90
Joshua Creek (2 stations)	4.48	1.58, 6.75

Table 3. Mass balance table for nitrate based on mean $\delta^{15}N$ in nitrate in sub-basin streams. See text for literature survey and data sources.

Sub-basin	Observed nitrate		Potential nutrient sources and stable nitrogen isotope ratios		Predicted contribution	
	$\delta^{15}N$, ‰	conc., mg/l	land use 1; expected ‰	land use 2; expected ‰	% nitrate from land use 1	% nitrate from land use 2
Charlie Creek	4.63	0.34	groundwater + fertilizer; 1.48	beef and dairy manure; 14	75%	25%
Joshua Creek	9.74	0.52	groundwater + fertilizer; 1.48	groundwater + sewage; 14	34%	66%
Horse Creek (upper half)	0.19	0.16	inorganic fertilizer; -1	natural lands; 4	76%	24%
Horse Creek (lower half)	8.22	0.30	groundwater + fertilizer; 1.48	beef and dairy manure; 14	46%	54%

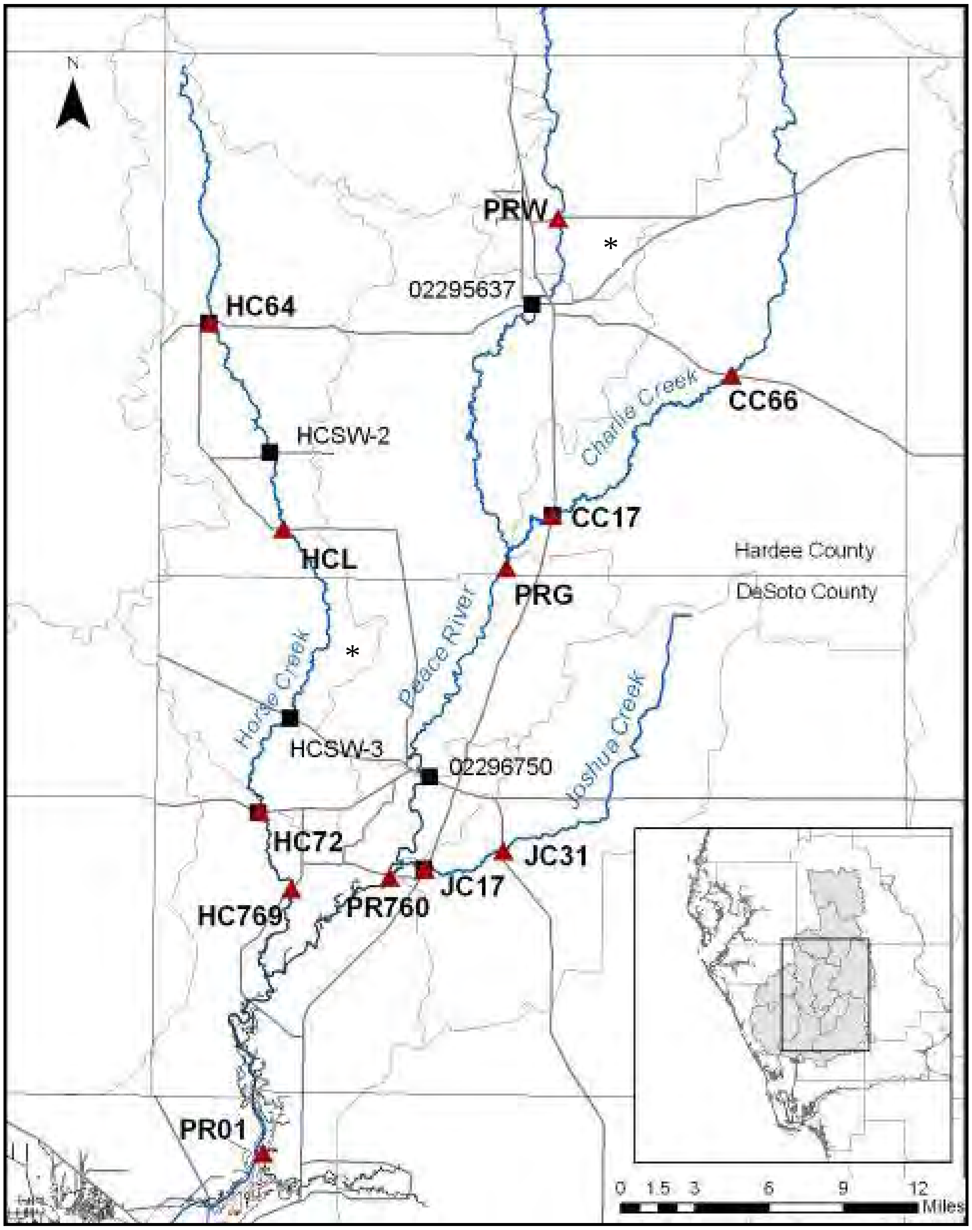

Figure 1. Locations sampled during this project (▲): PRW (Peace River at Wauchula); PRG (Peace River at Gardner); PR760 (Peace River at CR760, near Nocatee); PR01 (Peace River estuarine station); HC64 (Horse Creek at SR64); HCL (Horse Creek near Lily Community); HC72 (Horse Creek at SR72); HC769 (Horse Creek at CR769); CC66 (Charlie Creek at SR66); CC17 (Charlie Creek at US17); JC31 (Joshua Creek at SR31); JC17 (Joshua Creek at US17). Filled squares (■) identify supplemental water quality stations, see text. Symbol (*) in Hardee County approximates currently active domestic WWTP residuals land application site (as of 2007); symbol in DeSoto County approximates location of active industrial residuals land application site.

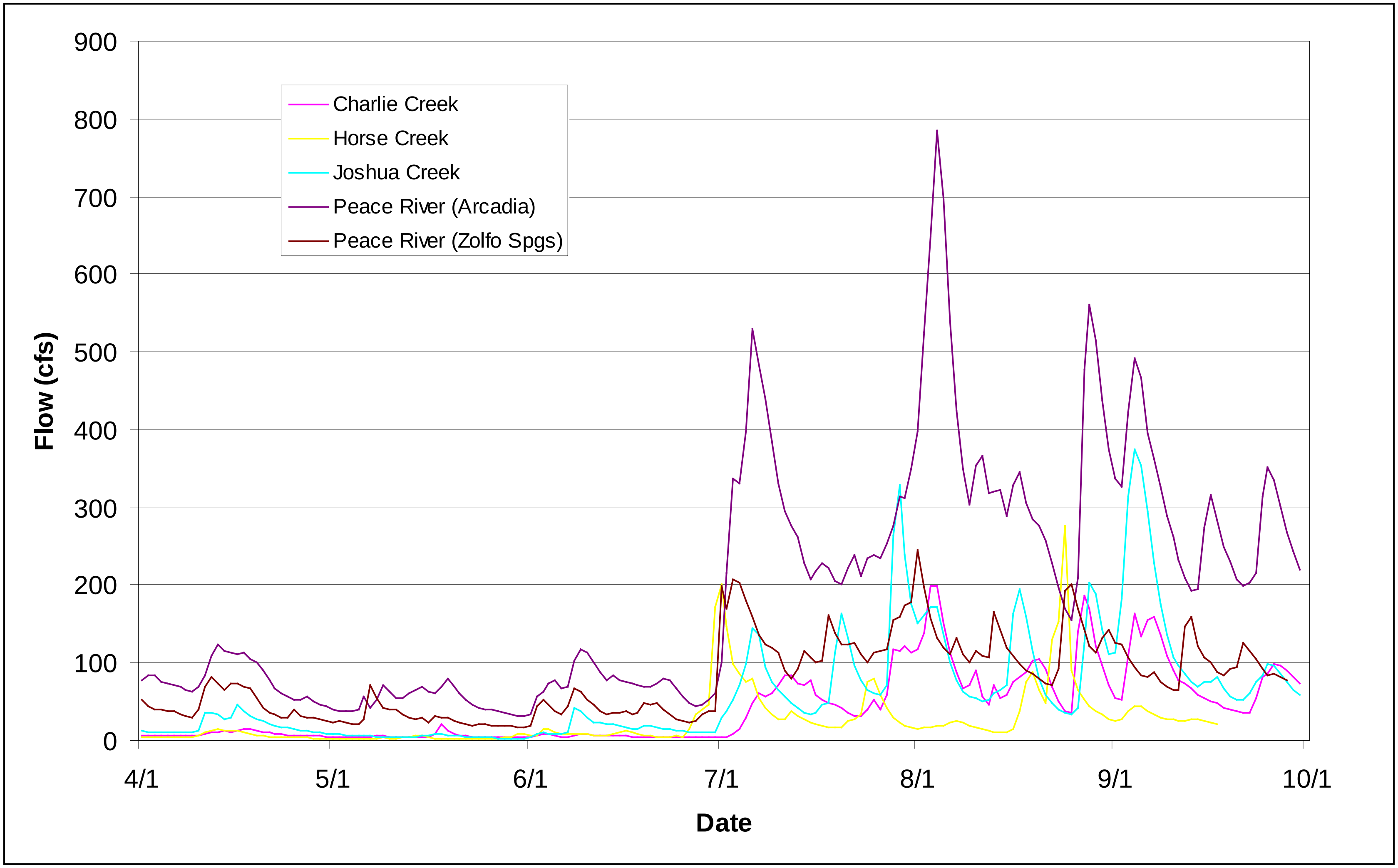

Figure 2. Flow (cfs) at five locations illustrating the difference between dry and wet seasons for 2007.

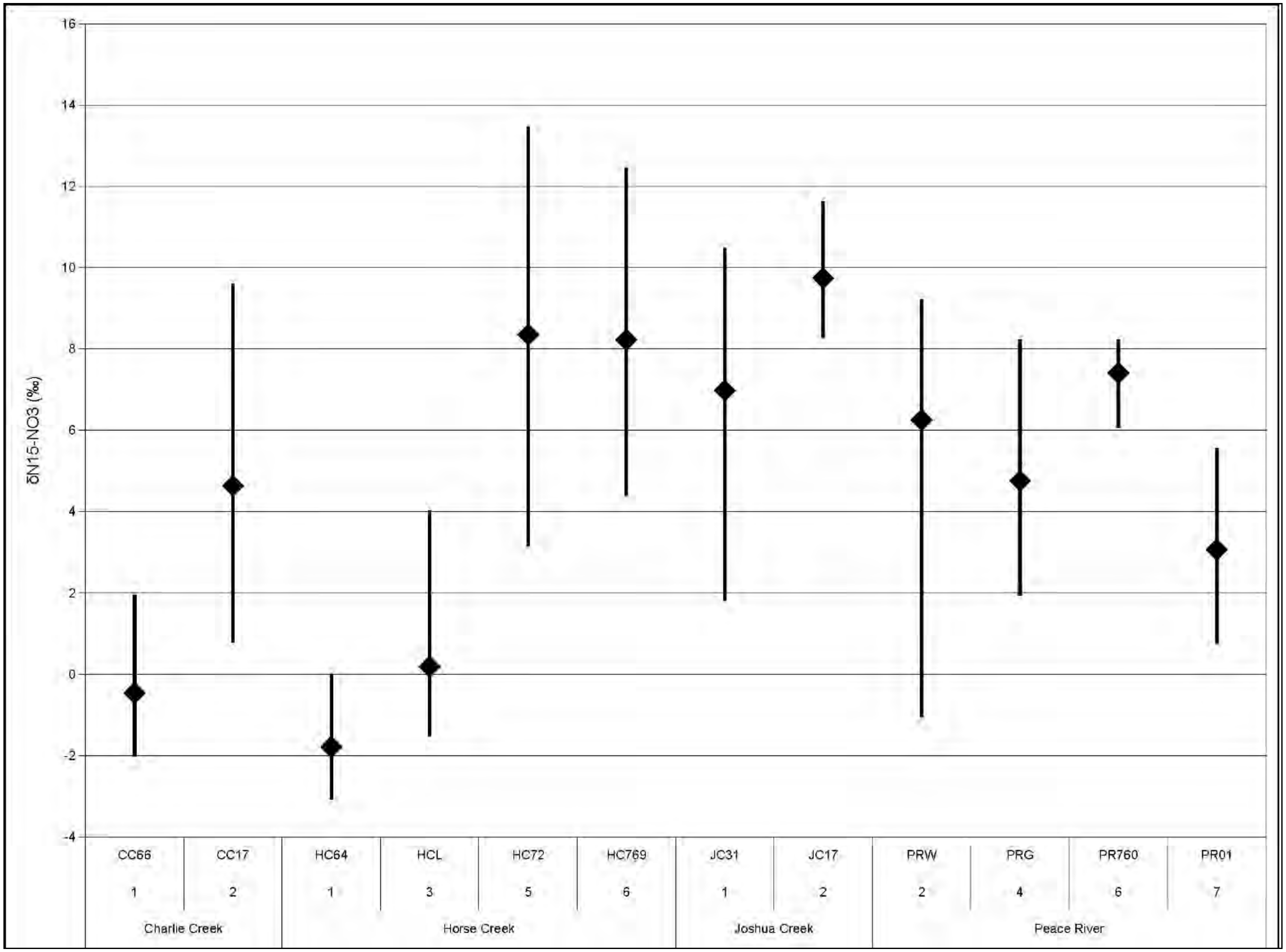

Figure 3. Mean, minimum, and maximum δ^{15}N in nitrate for all stations, arranged upstream to downstream within sub-basin groups.

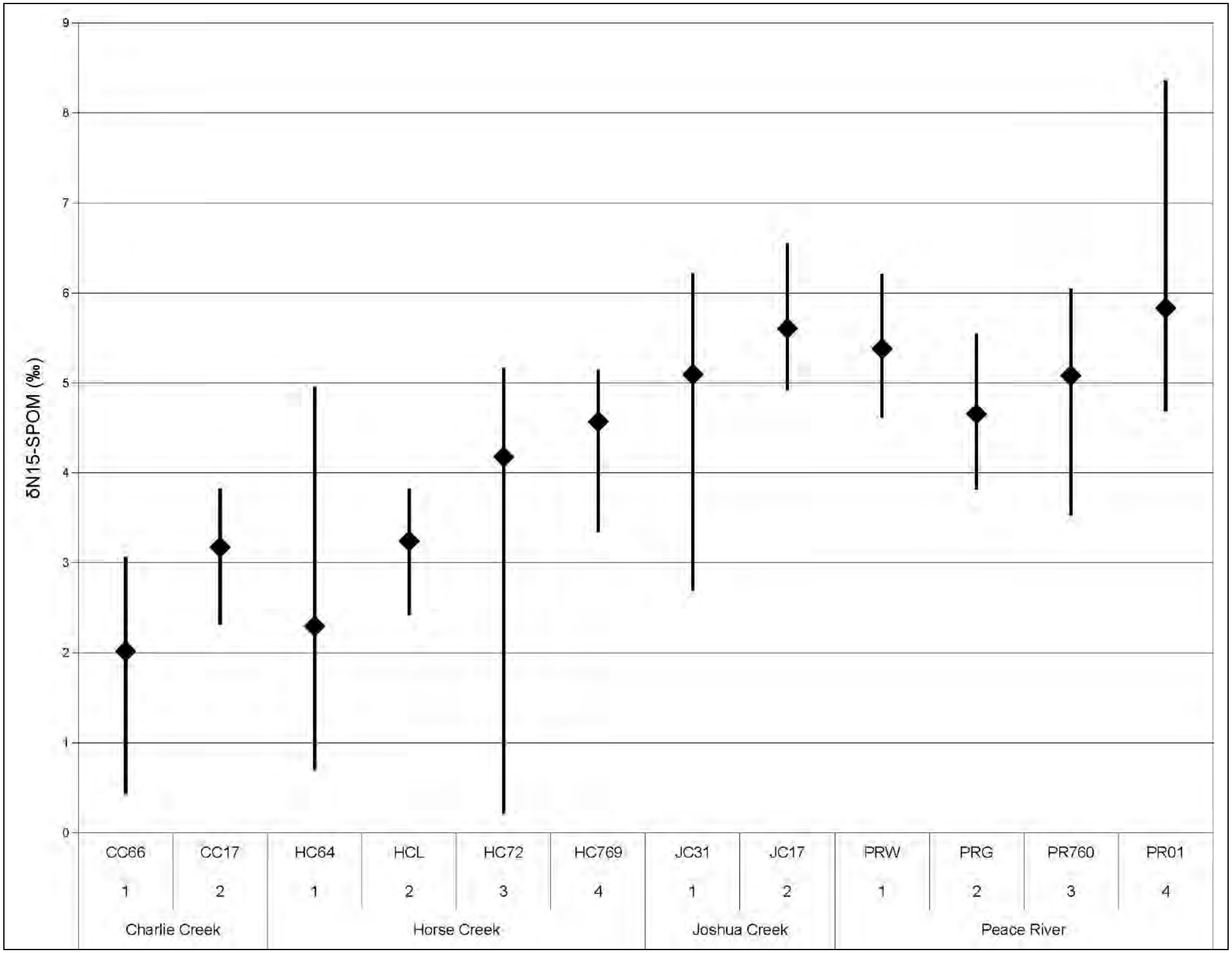

Figure 4a. Distributions of δ^{15}N in organic matter across stations grouped by sub-basins: suspended particulate organic matter (SPOM).

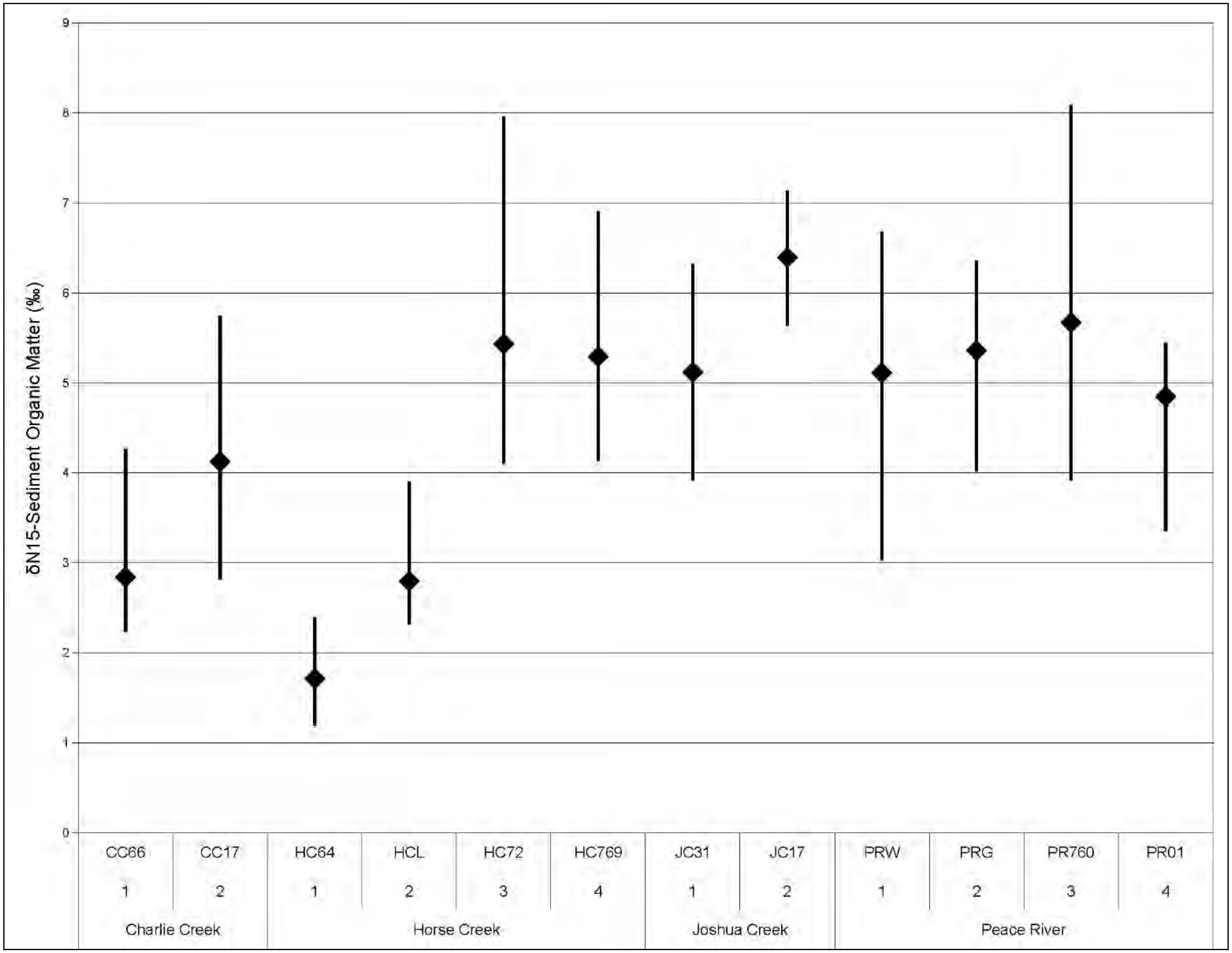

Figure 4b. Distributions of δ^{15}N in organic matter across stations grouped by sub-basins: sediment organic matter.

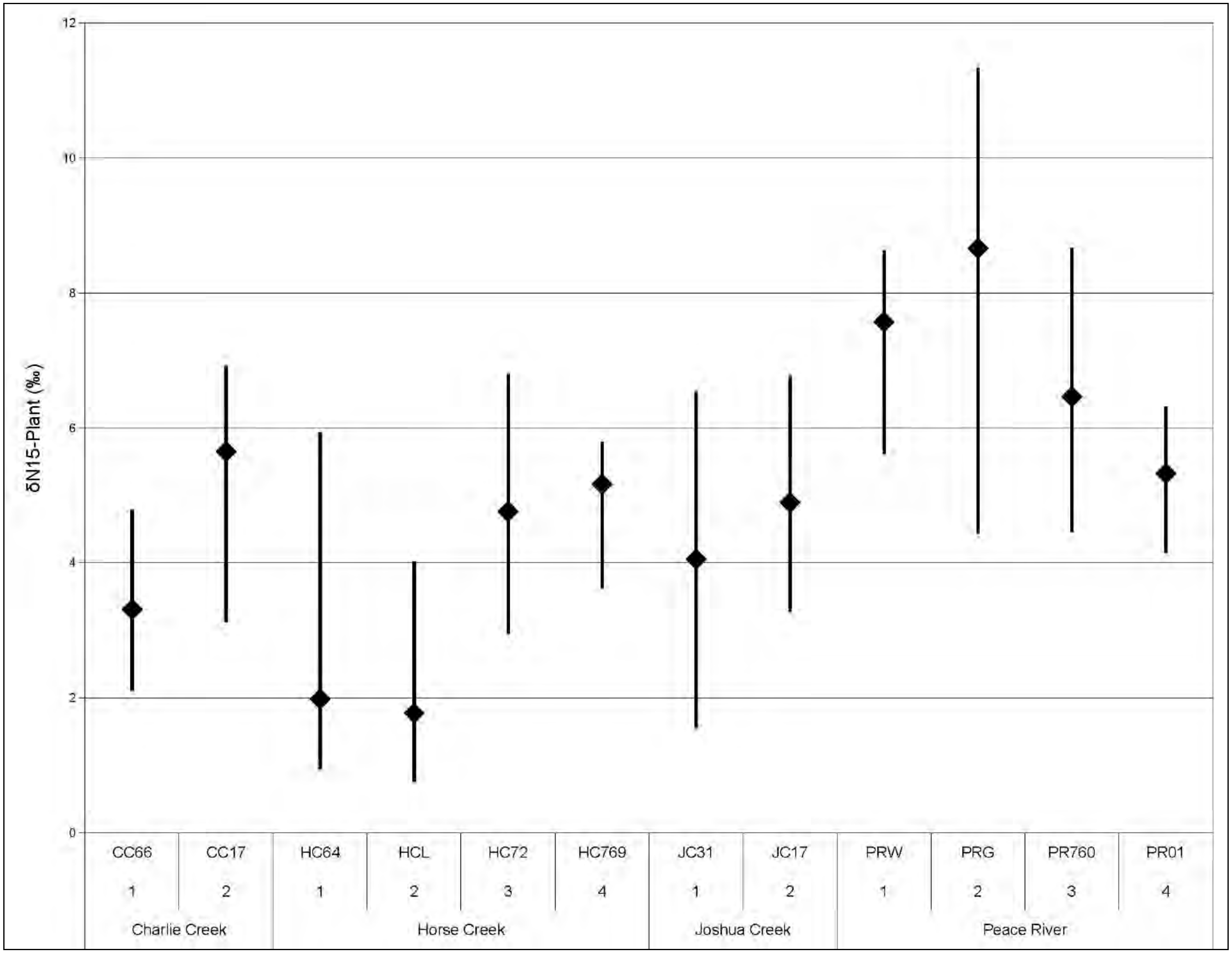

Figure 4c. Distributions of δ^{15}N in organic matter across stations grouped by sub-basins: stream side vascular plants.

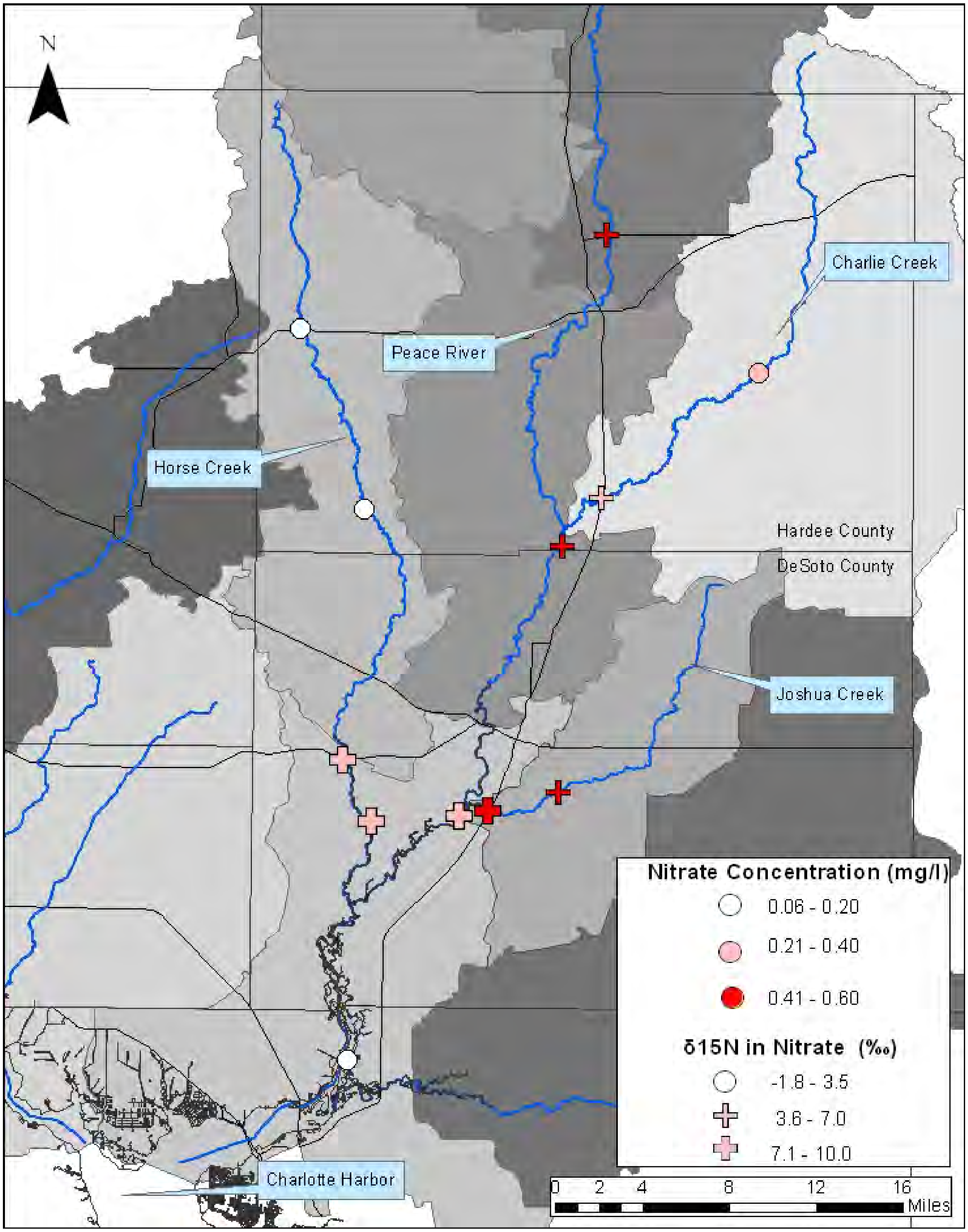

Figure 5. Relationships between mean estimated nitrate concentration and mean δ^{15}N in nitrate values; concentration is represented by color, stable isotope is represented by shape. Nitrate concentration estimates from various sources, see text.