

Inference of riverine nitrogen processing from longitudinal and diel variation in dual nitrate isotopes

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[1] Longitudinal and diel measurements of dual isotope composition ($\delta^{15}\text{N}$ and $\delta^{18}\text{O}$) in nitrate ($\text{NO}_3\text{-N}$) were made in the Ichetucknee River, a large ($\sim 8 \text{ m}^3 \text{ s}^{-1}$), entirely spring-fed river in North Florida, to determine whether isotopic variation can deconvolve assimilatory and dissimilatory removal. Comparing nitrate concentrations and isotope composition during the day and night we predicted (1) daytime declines in total fractionation due to low assimilatory fractionation and (2) diurnal variation in dual isotope coupling between 1:1 (assimilation) and 2:1 (denitrification). Five daytime longitudinal transects comprising 10 sampling stations showed consistent $\text{NO}_3\text{-N}$ removal (25–35% of inputs) and modest fractionation ($^{15}\epsilon_{\text{total}}$ between -2 and -6‰ , enriching the residual nitrate pool). Lower fractionation (by $\sim 1\text{‰}$) during two nighttime transects, suggests higher fractionation due to assimilation than denitrification. Total fractionation was significantly negatively associated with discharge, input $[\text{NO}_3\text{-N}]$, N mass removal, and fractional water loss. Despite well-constrained mass balance estimates that denitrification dominated total N removal, isotope coupling was consistently 1:1, both for longitudinal and diel sampling. Hourly samples on two dates at the downstream location showed significant diel variation in concentration ($[\text{NO}_3\text{-N}]$ amplitude = 60 to 90 $\mu\text{g N L}^{-1}$) and isotope composition ($\delta^{15}\text{N}$ amplitude = -0.7‰ to -1.6‰). Total fractionation differed between day and night only on one date but estimated assimilatory fractionation assuming constant denitrification was highly variable and implausibly large (for N, $^{15}\epsilon = -2$ to -25‰), suggesting that fractionation and removal due to denitrification is not diurnally constant. Pronounced counterclockwise hysteresis in the relationship between $[\text{NO}_3\text{-N}]$ and $\delta^{15}\text{N}$ suggests diel variation in N isotope dynamics. Together, low fractionation, isotope versus concentration hysteresis, and consistent 1:1 isotope coupling suggests that denitrification is controlled by NO_3^- diffusion into the benthic sediments, the length of which is mediated by riverine oxygen dynamics. While using dual isotope behavior to deconvolve removal pathways was not possible, isotope measurements did yield valuable information about riverine N cycling and transformations.

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1. Introduction

[2] Amplification of the global nitrogen cycle at least twofold during the twentieth century [Galloway *et al.*, 2004] has had deleterious effects on streams and river ecosystems [Dodds, 2006], estuaries [Smith, 2006] and, in some areas, human and animal health [Townsend *et al.*, 2003]. While there has been a marked increase in nitrogen export to the

coastal ocean ($\sim 65 \text{ Tg total N y}^{-1}$ [Seitzinger *et al.*, 2005]), the load applied to watersheds is substantially larger, indicating an estimated river network N removal efficiency near 75% [van Breemen *et al.*, 2002]. This important water purification process is distributed unevenly in space and time [McClain *et al.*, 2003], with removal occurring both in channels [Laursen and Seitzinger, 2004] and riparian zones [Sebilo *et al.*, 2003; Lowrance *et al.*, 1984], via assimilatory and dissimilatory pathways, and varying with environmental drivers (oxygen, discharge, light, temperature, organic matter concentrations) and stream order [Alexander *et al.*, 2000; Peterson *et al.*, 2001; Seitzinger *et al.*, 2002]. To predict and manage watershed N removal requires understanding rates, mechanisms and controls of N loss, which in turn necessitates methods that can be applied uniformly across stream order, geography and with sufficient intensity to capture natural variation.

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[3] Techniques for process-specific measurements of lotic N processing have focused on low-order streams [Tank *et al.*, 2008]; solute and isotope dosing studies [Hall *et al.*, 1998; Mulholland *et al.*, 2000] have yielded rates of and controls on N removal (assimilatory versus dissimilatory pathways [Böhlke *et al.*, 2004; Mulholland *et al.*, 2008]) and nitrification [Hamilton *et al.*, 2001]. Similar advances have lagged in larger rivers, primarily because of prohibitive costs of isotopic enrichment. Studies of N processing in high-discharge systems that have been done [e.g., Tank *et al.*, 2008] have used enrichment dosing techniques that draw inference from total removal of injected solutes that do not partition removal pathways; moreover, the logistics of large-volume dosing experiments constrains their utility for understanding removal variation in response to environmental or geomorphic controls.

[4] Natural stable isotope abundances are increasingly used for discerning sources and transformations of N [Kendall, 1998; Battaglin *et al.*, 2001; Sebilo *et al.*, 2006; Kendall *et al.*, 2007, Kellman and Hillaire-Marcel, 1998], offering a synoptic tool from which processes can be inferred in large and small rivers alike. Dual isotope measurements of nitrate ($\delta^{18}\text{O}_{\text{NO}_3}$ and $\delta^{15}\text{N}_{\text{NO}_3}$) have been applied to detecting variation in sources [Pellerin *et al.*, 2009], rates and locations of denitrification [Sebilo *et al.*, 2003; Chen *et al.*, 2009], nitrification [Sebilo *et al.*, 2006] and assimilation [Battaglin *et al.*, 2001; De Brabandere *et al.*, 2007; Deutsch *et al.*, 2009]. Since both dissimilatory and assimilatory pathways operate, and vary in their relative importance and absolute magnitude at diel, seasonal, and event scales, robust separation of N removal pathways that can be discerned from synoptic isotope sampling would aid efforts to understand and predict N processing in large rivers.

[5] Our objective in this study was to use dual nitrate isotopes to deconvolve removal processes in the spring-fed Ichetucknee River in north Florida where thermal, discharge and chemical stability, and high primary production yield coherent ecosystem-level signals (e.g., diel nitrate variation) that permit well constrained estimates of both assimilatory and dissimilatory N removal [Heffernan and Cohen, 2010]. Here we assess whether $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ measured longitudinally (on different days and nights) and diurnally can help partition N removal among pathways, and use repeated measurements to investigate controls on fractionation.

[6] While there are processes other than removal (primarily nitrification) that affect riverine nitrate isotopes, we focused on pathways of N removal, particularly biotic assimilation, which is intrinsically transient but may be significant at diel, seasonal or interevent time scales, and denitrification, which reduces nitrate to N_2 gas which evades to the atmosphere. Two lines of evidence were proposed to discriminate between dissimilatory and assimilatory removal: differential coefficients of ^{15}N enrichment, and differential coupling of ^{15}N and ^{18}O enrichment.

[7] Strong isotopic enrichment of N (reported as $^{15}\epsilon$, units of ‰ [Mariotti *et al.*, 1981] wherein negative values indicate preferential use of the lighter isotope resulting in enrichment of the residual water column pool) and O ($^{18}\epsilon$) isotopes in nitrate has been documented during denitrification, increasing the mass fraction of ^{15}N and ^{18}O in the residual nitrate pool ($^{15}\epsilon \sim -11$ to -30% , $^{18}\text{O} \sim -6$ to -18% [Sebilo

et al., 2006]). This range, typical of groundwater isotope enrichment, may not apply in surface waters, particularly where denitrification is nitrate limited (e.g., where diffusion limits benthic denitrification [Sebilo *et al.*, 2003] in low-redox wetland settings [Lund *et al.*, 1999]). Generally, however, high riverine fractionation has been observed (e.g., $^{15}\epsilon$ of -6 to -20% in the work by Ruehl *et al.* [2007] and -14.8% in the work by Chen *et al.* [2009]). Smaller but variable enrichment has been observed due to assimilation ($^{15}\epsilon = 0$ to -27% [Fogel and Cifuentes, 1993]) with fractionation declining with increased growth and decreased nutrient availability [Battaglin *et al.*, 2001]. Some studies [Lund *et al.*, 1999; Søvik and Mørkved, 2008] assume no fractionation due to assimilation, which may hold for emergent plants dominant in their study sites (though $^{15}\epsilon$ of -4.4% were estimated in riparian wetlands [Dhondt *et al.*, 2003]), but is unsupported for most aquatic systems. Montoya and McCarthy [1995] found higher fractionation in diatoms ($^{15}\epsilon$ of -9 to -12%) than for other phytoplankton ($^{15}\epsilon$ of -0.9 to -3.2%) suggesting that dominant primary producer is highly relevant to ecosystem-level fractionation. Notably, one study of vascular plants and epiphytic algae in a similar spring-fed river [De Brabandere *et al.*, 2007] observed $^{15}\epsilon$ between -0.9 to -3.2% , and no differences between primary producers. Based on these broad differences in fractionation, and assuming assimilation occurs during the day and denitrification is diurnally constant, we hypothesized that diel variation in isotope fractionation and nitrate flux could be used to deconvolve removal processes. The assumption of diurnally constant denitrification, which implies daytime dissolved oxygen enrichment does not inhibit the process, is also treated as a hypothesis. We predicted greater longitudinal removal of nitrate, the dominant form of N in the study site [Heffernan *et al.*, 2010], during the day (assimilation + denitrification), but reduced total fractionation vis-à-vis nighttime conditions because denitrification fractionates more strongly. Similarly, we predicted diel variation in $^{15}\epsilon$ would be lowest at peak assimilation, when $\delta^{15}\text{N}$ is enriched and nitrate depleted vis-à-vis night conditions.

[8] A second mode of discriminating N removal pathways focuses on differences in isotopic coupling (i.e., $\delta^{15}\text{N}_{\text{NO}_3}$ versus $\delta^{18}\text{O}_{\text{NO}_3}$) between assimilation and denitrification. Fractionation occurs in the same direction for both processes, with heavy isotope enrichment of the residual nitrate pool, but isotope coupling, measured as the slope of the association between $\delta^{15}\text{N}_{\text{NO}_3}$ versus $\delta^{18}\text{O}_{\text{NO}_3}$, differs. Specifically, during assimilation the slope is reported to be 1:1 (i.e., $^{15}\epsilon = ^{18}\epsilon$ [Granger *et al.*, 2004]), while the slope for denitrification is 1:2 (i.e., $^{15}\epsilon = 2 * \epsilon^{18}$ [Lehmann *et al.*, 2003]). Most studies reporting 1:2 coupling were for groundwater [Aravena and Robertson, 1998; Böttcher *et al.*, 1990], but both Ruehl *et al.* [2007] and Chen *et al.* [2009] provide supporting evidence for this mode of inference in rivers. Recent experimental evidence [Granger *et al.*, 2008] suggests 1:1 coupling during denitrification with both freshwater and marine denitrifiers; the conditions under which 1:2 coupling occurs remains an important uncertainty. The work by Ruehl *et al.* [2007] is notable for using ancillary evidence to confirm that other removal mechanisms (dilution, assimilation) cannot explain longitudinal depletion; their observation of 1:2 isotope coupling for

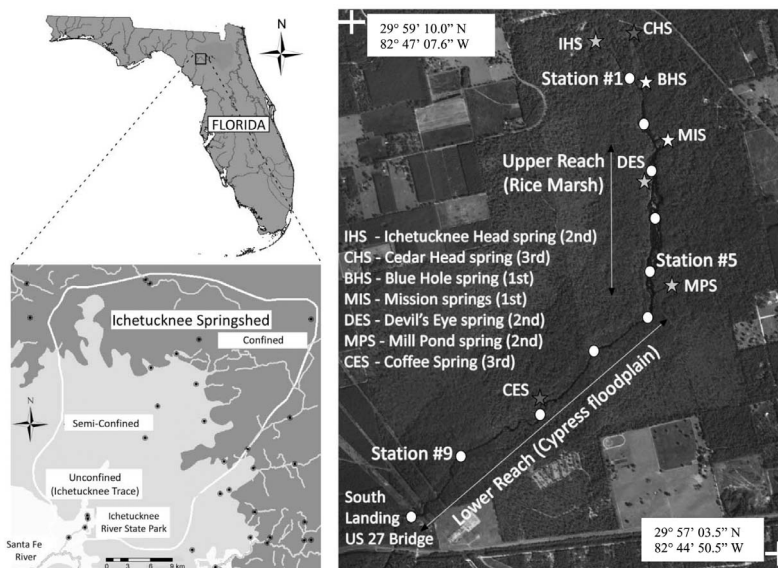


Figure 1. Study site showing the springshed (770 km²) in Columbia County, Florida, the six springs (stars) that feed the Ichetucknee River, longitudinal sample locations (n = 10, white circles with the upstream site 1), and downstream diel sampling location (at U.S. 27 bridge). Distinct morphologic zones (shallow/wide upper; deep/narrow lower) are marked.

denitrification is, thus, particularly robust. Based on all the literature evidence, we predicted isotope coupling would approach a 1:2 slope at night when denitrification is dominant, while the mixed removal process during the day would exhibit a slope between 1:1 and 1:2.

[9] Using these inferences to deconvolve N removal makes three assumptions. First, diel variation in nitrate concentration, and consequently in nitrate isotopes, is due to variation in assimilation only. That is, we assume denitrification is constant each day, and that fractionation due to denitrification is also constant. *Heffernan and Cohen* [2010] report significant interday variation in denitrification, but used correlative evidence to conclude that within-day variation in denitrification rates is negligible. The data collected here permit a formal test of this assumption. Second, we assume assimilation at night is negligible; estimates of N assimilation from diel nitrate variation were consistent with GPP stoichiometry and biomass turnover only when nighttime autotrophic uptake was assumed zero [*Heffernan and Cohen*, 2010]. Third, we assume that lateral water inputs of unknown isotopic composition are minimal. Despite piezometric (M. Kurz, unpublished data, 2011) and conservative solute [*de Montety et al.*, 2011] data that suggest some diffuse lateral groundwater inputs in the upper reaches, total springs discharge is typically higher (10–20%) than measured downstream flows, with the losses occurring below the zone of springs discharge (station 6, Figure 1). We assumed the Ichetucknee is a losing river only below the zone of spring discharge, simplifying inference of longitudinal processing.

[10] Factors controlling temporal variation in fractionation are essential for interpreting synoptic measurements. Measures of input nitrate concentrations, discharge (inputs and river water loss), and N removal, along with dual isotope inference, allow enumeration of covariates with fractionation. Several studies have examined temporal variation in

fractionation. *Ruehl et al.* [2007] report strong discharge dependence on both N removal (reduced at higher discharge) and fractionation (greater at higher discharge). Similarly, *Chen et al.* [2009] report seasonal variation in fractionation due to denitrification, with fractionation increasing under high load conditions and in response to temperature and discharge. Our expectations in this stable spring-fed river were that fractionation would increase with discharge due to increased interaction with hyporheic and riparian sediments, and decrease at lower concentrations and with greater N removal signaling more complete processing of the available nitrate.

2. Methods

2.1. Study Site

[11] The Ichetucknee River is an entirely spring-fed tributary of the Santa Fe River and part of the Suwannee River basin in North Florida, United States. The 770 km² springshed recharges water to the Upper Floridan Aquifer, which discharges to 6 major spring vents in the southern part of the basin where the carbonate aquifer is unconfined (Figure 1) [*Scott*, 1992]. Daily discharge of the six major springs and the Ichetucknee River at U.S. 27 is available since February 2002, over which time flow has varied only threefold; low discharge variability, and the absence of episodic scouring of accumulated organic material, is one reason spring rivers are useful model systems. Over that period, downstream discharge at U.S. 27 averaged 8.6 m³ s⁻¹, ~11% less than the combined flow of the springs (9.6 m³ s⁻¹); we note, however, that diffuse groundwater inputs in the upper river may still be significant even though the lower river below the spring inputs is a losing river.

[12] Channel morphology and water chemistry change over the 8 km length of the river in response to sequential mixing of spring vents. Within 1 km of the Head Spring

(median discharge = $1.3 \text{ m}^3 \text{ s}^{-1}$), the river is fed by water from Blue Hole ($3.6 \text{ m}^3 \text{ s}^{-1}$) and Cedar Head Springs ($0.3 \text{ m}^3 \text{ s}^{-1}$), followed by the Mission Springs complex ($2.6 \text{ m}^3 \text{ s}^{-1}$) and Devil's Eye spring ($1.4 \text{ m}^3 \text{ s}^{-1}$) (Figure 1). To the point where Mission Springs enters, the river is of intermediate width (mean = 27 m), shallow (mean depth = 0.85 m) and slow moving (mean velocity = 0.22 m s^{-1}). Over the next 1000 m, the river passes through an area known as the Rice Marsh reach where it widens substantially (mean width = 65 m). Flow is primarily routed through a deeper (mean depth = 1.0 m) thalweg that is 20–25 m wide, but flow is also evident throughout a shallower (mean depth = 0.4 m) highly vegetated zone that remains wetted during all but the most extreme low-flow periods. At the end of the rice marsh, two more springs (Grassy Hole: $0.2 \text{ m}^3 \text{ s}^{-1}$, and Mill Pond: $0.8 \text{ m}^3 \text{ s}^{-1}$) enter the river, and the channel narrows substantially (mean width = 24 m), deepens (mean depth = 1.2 m) and velocity increases (mean velocity = 0.35 m s^{-1}). The channel is confined by a wide floodplain (75–200 m) that is inundated episodically by backwater effects of stage variation in the downstream Santa Fe River, 8 km from the headspring; the boundary of Ichetucknee River State Park at the U.S. 27 bridge, 5 km from the headspring, is the downstream extent of this study. Based on these 10 measurements of channel width and an estimate of thalweg length from aerial imagery, we estimated the benthic surface area to be $175,000 \text{ m}^2$. The median hydraulic residence between Blue Hole Spring and downstream is 6 h, and conservative tracer breakthrough curve analysis suggests that less than 5% of the water resides in the river longer than 9 h, presumably in the Rice Marsh reach [Hensley, 2010].

[13] Water chemistry varies across springs due to different contributing areas, flow paths and residence times, but remains remarkably constant over time within springs [Martin and Gordon, 2000]. Elevated nitrate-N concentrations, up to 16 times reported background levels (i.e., pre-development concentrations of $\sim 0.05 \text{ mg L}^{-1}$ [Katz, 1992]), are found in all springs, but are particularly significant in Head, Cedar and Blue Hole (0.77, 0.82, 0.70 mg L^{-1} , respectively), with Mission, Devils and Mill Pond moderately lower (0.52, 0.55, 0.41 mg L^{-1} , respectively). Based on isotopic and mass balance evidence, mineral fertilizer, likely applied to row crop agriculture, pasture and managed forests, is the principal N source [Katz et al., 2009]; the springshed also includes Lake City (pop. 10,000), and many septic tanks. Monthly water chemistry measurements over 15 months between 2007 and 2008 showed a mean coefficient of variation for $[\text{NO}_3\text{-N}]$ of 7% across springs and autocorrelation at 1 month lag of +0.82, supporting our assumption of constant boundary inputs over any given sampling event.

[14] Previous work [Heffernan et al., 2010; Heffernan and Cohen, 2010] indicated that denitrification dominates N removal. A high-resolution mass balance based on flow-weighted $\text{NO}_3\text{-N}$ concentrations from the springs (measured monthly) and observed diel variation in downstream river concentrations (measured hourly) yielded daily assimilation and denitrification that aligned with long-term mass balance calculations using archival data (from EPA STORET) [Heffernan and Cohen, 2010]. Multiple methods to estimate

autotrophic N demand from gross primary production suggest denitrification comprises between 75 and 85% of observed longitudinal N removal (20 year mean total removal = $0.77 \text{ g N m}^{-2} \text{ d}^{-1}$ [Heffernan et al., 2010]). Heffernan et al. [2010] note that assimilated N is either exported as particulate organic matter, likely a small flux based on river suspended material concentrations, or mineralized and nitrified to nitrate since (1) dissolved TKN is low and constant along the entire study reach and (2) ecosystem storage in this pulse-free river is assumed to be continuously near equilibrium. Nitrification rates have not been measured directly. Variation in assimilatory and dissimilatory N removal rates was similar across seasons [Heffernan and Cohen, 2010], suggesting strong coupling between rates of organic matter production and heterotrophic mineralization.

2.2. Field Sampling

[15] Filtered water samples were collected from each of the 6 springs monthly between March 2007 and March 2008, and then again during the spring and fall of 2009. Samples were also collected from a longitudinal transect consisting of 10 fixed locations (Figure 1) in the early afternoon (between 2:00 P.M. and 4:00 P.M. EST) on 5 days (September 2007, March 2008, March 2009, October 2009 and November 2009). The purpose of these transects was not to fully characterize the spatial and temporal variation in isotopic behavior, but rather to evaluate the consistency of the fractionation and coupling signal. That said, we were able to collect data from two seasons (fall and spring; summer sampling was not possible), and, despite lingering regional drought, a range of discharge conditions (7 to $8.4 \text{ m}^3 \text{ s}^{-1}$). Predawn samples were collected at each station the morning following daytime sampling in October 2009 and November 2009. Measurements of pH, dissolved oxygen, and temperature were obtained using a YSI field sonde (YSI 650, Yellow Springs OH) that was calibrated prior to each field day.

[16] Water samples were also collected hourly over a 24 h period using an ISCO 6700 autosampler at the downstream location (U.S. 27 bridge) on two occasions (March 2009, November 2009). Discrete samples were collected by hand at the start and finish of each 24 h period to control for sampling device or holding time effects; based on evidence of contamination, a third day (October 2009) of hourly samples was excluded from further analysis.

[17] All samples for $\text{NO}_3\text{-N}$ concentrations and dual isotope ($\delta^{15}\text{N}$ and $\delta^{18}\text{O}$) composition were collected in acid-washed 150 mL brown polyethylene bottles and frozen until analysis. Nitrate concentrations were measured within 28 days using second-derivative UV spectroscopy (Aquamate UV-Vis spectrometer); these waters are naturally low in UV-absorbing dissolved organic matter, minimizing interference due to color. Because discharge from the springs enter at different locations along the upper river, the expected concentrations assuming mixing only were computed based on the flow weighted average concentration from springs that discharge upstream of each sampling location; longitudinal removal was evaluated vis-à-vis this quantity.

2.3. Isotope Measurements

[18] Nitrate isotopes were measured at the University of Florida, Department of Geological Sciences, using the bacterial denitrifier method [Sigman *et al.*, 2001; Casciotti *et al.*, 2002] whereby nitrate was quantitatively converted to N₂O by the bacteria *Pseudomonas aureofaciens*. The $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ of the N₂O produced was measured on a Thermo Delta-Plus XP isotope ratio mass spectrometer using the GasBench interface and a continuous flow of helium. Isotopic composition was reported using the δ notation relative to air (for ^{15}N) and VSMOW (for ^{18}O). International ^{15}N standard (IAEA-N3 $\delta^{15}\text{N} = +4.72\text{‰}$) and ^{18}O standards (USGS-34 = -27.9‰ , USGS-35 = $+25.5\text{‰}$) were included in each batch along with laboratory duplicates to estimate overall analytical precision of $\pm 0.2\text{‰}$ for $\delta^{15}\text{N}$ and $\pm 0.6\text{‰}$ for $\delta^{18}\text{O}$.

[19] The isotopic composition at each sampling location (j) that would have occurred assuming mixing only (D_j) was computed based on daily gage-estimated discharge (Q_i), and measured nitrate concentration (C_i) and isotopic composition (d_i) for each spring (i) during that month (not necessarily on our sampling date). Low temporal variation in spring nitrate concentrations and isotope composition (CV $\delta^{15}\text{N} = 3\%$, 7% , 3% , 2% , 4% , and 13% for Head, Cedar, Blue, Mission, Devils and Mill Pond, respectively, over 18 monthly samples) supported using this asynchronous input data. The initial estimate of the flux-weighted mixing-only isotope value ($D_{\text{FW},j}$) at any location, j, was computed based on all upstream springs inputs:

$$D_{\text{FW},j} = \frac{\sum_i Q_i C_i d_i}{\sum_i Q_i C_i} \quad (1)$$

However, this formulation fails to consider apparent fractionation that occurs because of the particular spatial arrangement of springs in the Ichetucknee system. Specifically, the first spring (Ichetucknee Head Spring) is isotopically lightest, and subsequent springs are increasingly heavy. Because springs that discharge further upstream have a longer residence time in the river than those entering further downstream, more of their N is removed at any given point. As such, even in the absence of a fractionating removal process, apparent fractionation would occur because the isotopically lighter inputs have been processed for longer. To control for this effect, we adjusted the flux-weighted springs mixture by the expected fraction of N remaining ($R_{i,j}$) from each spring input, i, at each location, j, for all dates sampled. To determine $R_{i,j}$ at each station and for each spring, we imputed a constant fractional nitrate removal rate (U) with distance based on the observed aggregated decline in $[\text{NO}_3\text{-N}]$ such that the fraction of nitrate remaining ($R_{i,j}$) from each spring input was a function of a station's distance downstream of that spring ($x_{i,j}$). That is,

$$R_{i,j} = 1 - (x_{i,j} * U) \quad (2)$$

The expected isotope value ($D_{\text{FWA},j}$) at each station, vis-à-vis which fractionation was computed, was obtained as follows:

$$D_{\text{FWA},j} = \frac{\sum_i Q_i C_i d_i R_{i,j}}{\sum_i Q_i C_i R_{i,j}} \quad (3)$$

where values at each location, j, are as in equation (1) except for the adjustment based on the fraction of N remaining ($R_{i,j}$, range from 0 to 1). The effects of this adjustment for apparent fractionation were small, increasing the value of $D_{\text{FWA},j}$ over the $D_{\text{FW},j}$ at the most downstream location by a maximum of 0.08‰. However, this adjustment did affect inference of longitudinal and diurnal fractionation by up to 10% of the value without correction. Note that we similarly corrected for apparent fractionation in $^{18}\text{O}_{\text{NO}_3}$.

2.4. Data Analysis

[20] Longitudinal enrichment factors ($^{15}\epsilon$ and $^{18}\epsilon$ for N and O in nitrate, respectively [Mariotti *et al.*, 1981]), were determined from the slope of a regression line between the natural logarithm of the remaining nitrate in the water column versus isotope abundances compared to expected values without fractionation (i.e., $\Delta\delta^{15}\text{N}_j = \delta^{15}\text{N}_j - D_{\text{FWA},j}$). The NO₃ remaining was indexed to measured inputs as Ln $([\text{NO}_3\text{-N}]_i / [\text{NO}_3\text{-N}]_0)$, where $[\text{NO}_3\text{-N}]_i$ is the concentration at location i and $[\text{NO}_3\text{-N}]_0$ is the expected concentration based on spring input mixing only. Longitudinal isotope coupling between $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ was evaluated using ordinary least squares (OLS) regression to obtain slope estimates; significant deviation of the fitted slope (β) from 1.0 was determined by evaluating a test statistic, $(\beta - 1 / \text{SE})$, where SE is the slope standard error using a t distribution. OLS goodness of fit (r^2) described the strength of coupling. Associations between inferred enrichment factors and environmental covariates (total springs discharge, longitudinal water loss, longitudinal N loss, flow-weighted nitrate inputs) were also evaluated using OLS regression; due to low power, our comparison of day versus night longitudinal fractionation was qualitative. Total N removal was estimated from the longitudinal decline in nitrate mass, assuming that any water lost from the river between the springs and downstream sampling location had the same nitrate removal as was observed in water remaining in the channel. These total loss rates matched closely with previously estimates [Heffernan *et al.*, 2010; Heffernan and Cohen, 2010]. Further, on 2 days when both day and night (predawn) longitudinal transects were sampled, denitrification was estimated from incremental daytime mass removal over nighttime, assuming denitrification is diurnally constant, and observed day versus night isotope changes (i.e., $\Delta\delta^{15}\text{N}$ for night versus day).

[21] Diel isotope coupling was evaluated over 24 h and for both day and night segments on 2 days; a third day of diel samples were obtained (October 2009), but were omitted from this study because of apparent autosampler contamination. The first day (March 2009) was cloudy while the second (November 2009) was sunny; cloud cover was expected to affect both primary production that day and denitrification the subsequent day [Heffernan and Cohen, 2010]. Hourly variation in total isotopic enrichment was determined as the difference between springs inputs of nitrate and isotope abundance versus downstream observations. These diel fractionation values were compared to estimates from simultaneous longitudinal transects. For diel time series, isotope enrichment due to denitrification ($^{15}\epsilon_{\text{den}}$) was estimated from total $^{15}\epsilon$ values observed between 10:00 P.M. and 7:00 A.M. EST (i.e., when

Table 1. Summary of Ichetucknee Springs Inputs During the Study Period

Spring	Flow ($\text{m}^3 \text{s}^{-1}$)					[NO ₃ -N] (mg N L^{-1})				
	Sep 2007	Mar 2008	Mar 2009	Oct 2009	Nov 2009	Sep 2007	Mar 2008	Mar 2009	Oct 2009	Nov 2009
Ichetucknee	1.30	1.36	1.27	1.42	1.45	0.78	0.82	0.75	0.84	0.82
Cedar	0.15	0.14	0.15	0.16	0.15	0.83	0.83	0.82	0.82	0.76
Blue Hole	3.11	2.91	2.72	2.18	2.15	0.72	0.68	0.67	0.64	0.63
Mission	2.43	2.63	2.26	2.49	2.46	0.49	0.50	0.48	0.45	0.45
Devils Eye	1.44	1.22	1.33	0.99	0.98	0.49	0.50	0.45	0.48	0.47
Mill Pond	0.62	0.82	0.59	0.59	0.57	0.37	0.35	0.37	0.25	0.25
River at U.S. 27 ^a	7.58	8.43	7.13	7.30	7.02	0.61	0.59	0.57	0.56	0.55

Spring	$\delta^{15}\text{N}_{\text{NO}_3}$ (‰)					$\delta^{18}\text{O}_{\text{NO}_3}$ (‰)				
	Sep 2007	Mar 2008	Mar 2009	Oct 2009	Nov 2009	Sep 2007	Mar 2008	Mar 2009	Oct 2009	Nov 2009
Ichetucknee	3.8	3.7	3.2	3.2	3.5	9.2	9.3	5.7	5.7	5.3
Cedar	3.7	3.7	3.8	3.1	3.6	9.5	9.7	6.4	5.7	4.9
Blue Hole	4.3	4.2	4.2	4.3	4.3	10.2	9.0	6.6	6.7	6.0
Mission	6.8	6.8	7.2	8.4	8.3	13.5	12.4	10.3	11.3	11.2
Devils Eye	7.3	6.8	9.6	10.9	11.7	13.4	12.4	11.5	15.3	14.9
Mill Pond	7.3	8.3	12.5	17.5	17.1	13.4	14.5	14.2	13.4	13.5
River at U.S. 27 ^a	5.4	5.3	5.8	6.1	6.1	11.5	10.8	8.3	8.6	8.6

Spring	DO (mg L^{-1})					Temperature ($^{\circ}\text{C}$)				
	Sep 2007	Mar 2008	Mar 2009	Oct 2009	Nov 2009	Sep 2007	Mar 2008	Mar 2009	Oct 2009	Nov 2009
Ichetucknee	4.8	4.1	3.9	4.2	3.8	22.8	21.9	21.8	21.8	21.7
Cedar	3.7	3.1	3.2	3.1	3.2	22.0	21.7	21.4	21.7	21.6
Blue Hole	2.2	1.8	1.8	2.3	2.0	21.8	21.7	21.8	21.8	21.8
Mission	2.0	0.5	0.7	0.5	0.5	22.1	21.9	22.0	21.8	21.8
Devils Eye	-	0.7	0.9	0.4	0.4	-	22.0	21.9	21.7	21.8
Mill Pond	0.4	0.5	0.5	0.3	0.4	22.1	21.9	22.1	21.9	21.9
River at U.S. 27 ^a	2.0	1.5	1.6	1.7	1.6	22.1	21.8	21.9	21.8	21.8

^aChemical concentrations for the river at U.S. 27 are flow weighted (for NO₃-N, dissolved oxygen, and temperature) and flux-weighted inputs (for isotope values), not observations.

assimilation is negligible). Mass loss due to denitrification was estimated from the same period, and both denitrification removal and fractionation were initially assumed constant. Assimilation was estimated from additional daytime mass removal; fractionation due to assimilation ($^{15}\epsilon_a$) was estimated from the difference between isotope values expected with denitrification alone ($^{15}\epsilon_{\text{den}}$) and observed hourly isotope values during the day.

3. Results

[22] Springs inputs to the Ichetucknee were remarkably constant (Table 1). Flow varied over the period of study (March 2007 to November 2009) between 7 and 8.5 $\text{m}^3 \text{s}^{-1}$, while monthly measurements of flow weighted nitrate concentration varied between 0.61 and 0.56 mg L^{-1} and flux-weighted isotopic composition ($D_{\text{FWA},j}$; equation (3)) varied between 5.4 and 6.1‰ (for $\delta^{15}\text{N}$) and 8.1 and 11.5 (for $\delta^{18}\text{O}$) (Table 1; solid lines in Figures 2b–2h). The coefficient of variation within springs averaged less than 10% for most attributes (temperature, pH, conductivity, nitrate, $\delta^{15}\text{N}$, and $\delta^{18}\text{O}$) except for dissolved oxygen, (mean CV of 21% across springs), and $\delta^{15}\text{N}$ in Mill Pond spring (CV = 13%). In short, upstream boundary conditions were constant, validating use of spring vent conditions measured within 10 days of diel and longitudinal sampling events as upstream inputs. Modest differences in the nitrate and isotope contribution of the springs, principally driven by temporal variation in discharge (Table 1), was evident in subtle shifts in the shape of

solid lines denoting flow-weighted nitrate and flux-weighted isotope inputs (Figures 2b–2h).

[23] Longitudinal samples show marked and consistent depletion of nitrate and enrichment of ^{15}N (Figures 2b–2h; black circles are observed nitrate, gray diamonds are observed $\delta^{15}\text{N}$). An important component of the general trend observed across all 7 transects of declining nitrate concentrations and increasing ^{15}N composition arises from the spatial arrangement of springs, with the highest concentrations and lowest $\delta^{15}\text{N}$ values in the upper springs; however, biological activity clearly caused deviations from values expected based on mixing (e.g., solid lines, Figure 2). Removal and isotope enrichment were modestly reduced during nighttime sampling events when compared to preceding days in October 2009 (Figures 2e and 2f) and November 2009 (Figures 2g and 2h).

[24] Total N removal was between 27% (October 2009) and 35% (March 2008) during the day (Figure 2). Given measured discharge on each day, this corresponds to total removal between 8.8 kg/h (1.1 $\text{g m}^{-2} \text{h}^{-1}$) in March 2008 and 5.5 kg/h (0.75 $\text{g m}^{-2} \text{h}^{-1}$) in October 2009. Nighttime removal observed in October and November 2009 suggests that denitrification accounted for 78 and 76% of total removal (0.59 and 0.60 $\text{g m}^{-2} \text{h}^{-1}$), respectively, consistent with long-term mass balance estimates of the relative contributions of assimilation (19%) and denitrification (81%) to overall removal [Heffernan *et al.*, 2010]. Based on longitudinal profiles, most nitrate removal occurred between stations 3 and 6, which is in the broad, shallow Upper Reach (Figure 1).

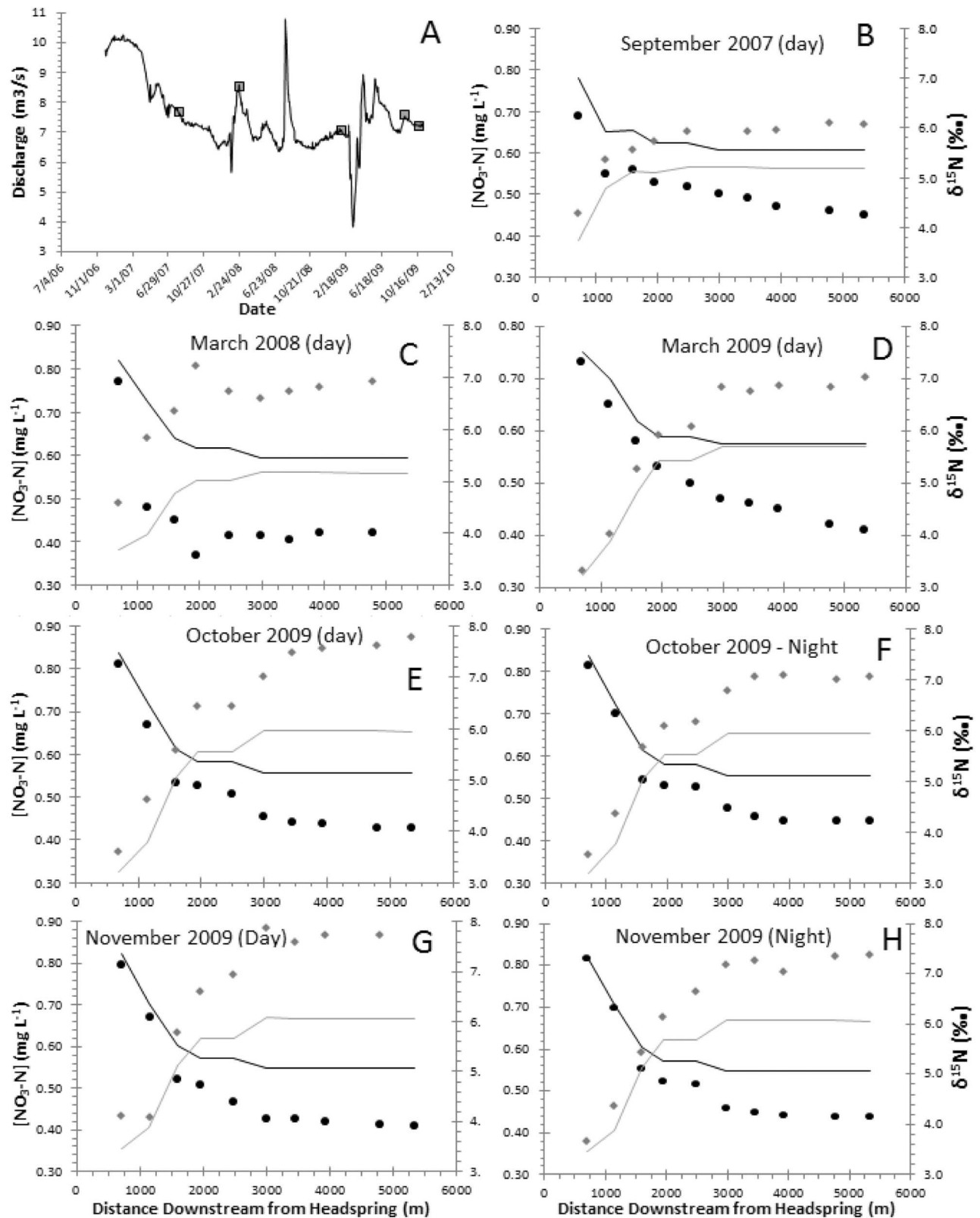


Figure 2. (a) Measured downstream discharge (black line) showing longitudinal sampling events (gray diamonds); hourly sampling was done in March and November 2009. (b–h) Longitudinal changes in [NO₃-N] (black circles) and δ¹⁵N (gray diamonds) are shown from seven events (five day, two night). Spring inputs assuming mixing only are shown for [NO₃-N] (black line) and δ¹⁵N (gray line); deviation between observations and this line indicate biological processing.

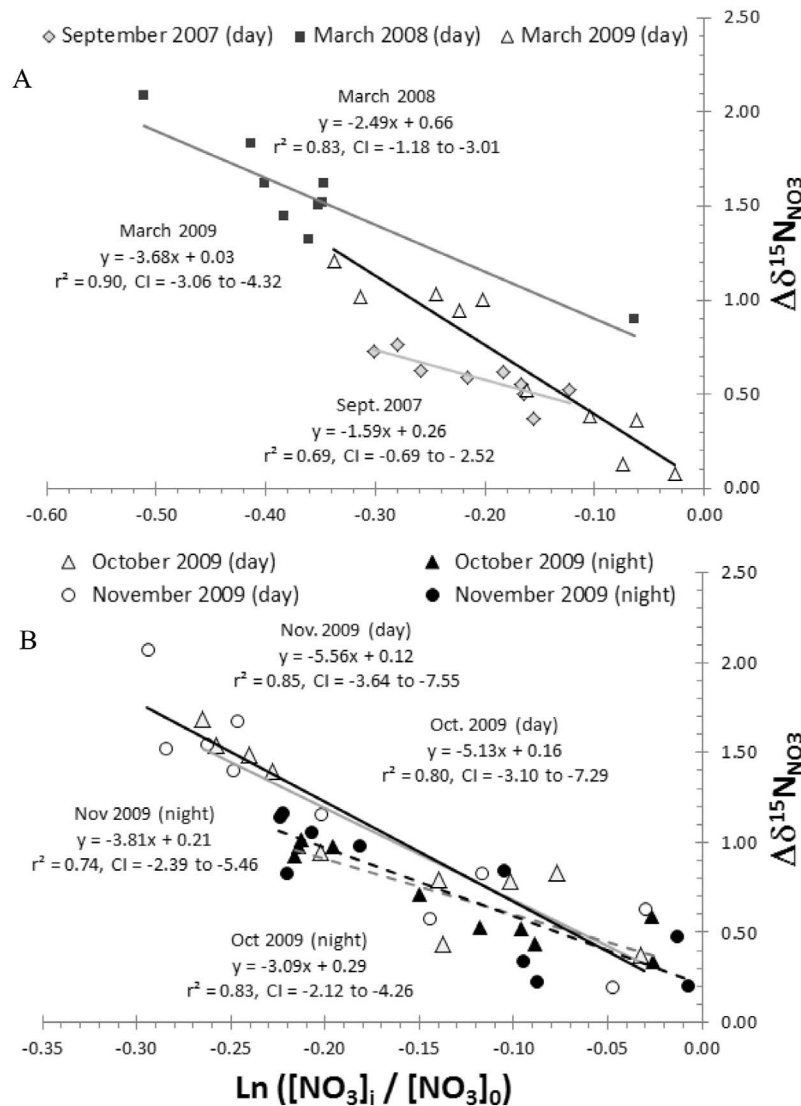


Figure 3. (a and b) Isotope enrichment ($\Delta\delta^{15}\text{N}$ between observations and flux-weighted spring inputs, adjusted for apparent fractionation) of residual $[\text{NO}_3\text{-N}]$ from seven transects (five day, solid lines; two night, dashed lines); fitted regression slopes are enrichment factors (ϵ^{15} in ‰), and 95% confidence intervals (CI) are given for each estimate.

[25] Isotopic enrichment with N removal (Figure 2) suggested fractionation during all sampling events. Estimated enrichment factors ($^{15}\epsilon$; fitted line slopes) for the 5 daytime transects varied between -1.59‰ (September 2007) and -5.56‰ (November 2009) (Figures 3a and 3b). Intercept values were statistically different from zero only for September 2007 and March 2008 when longitudinal removal was unusually strong in the upper river (stations 1–3; Figures 2c and 2d, respectively). Omitting the first station data point (rightmost symbol), which exerted particularly high leverage in March 2008, fractionation increased to -1.9 and -3.4‰ in September 2007 and March 2008, respectively. Intercept estimates declined by 50% and 20%, respectively, but were still significantly different from 0. Forcing the fitted line through 0 yields higher fractionation factors (-2.6 and -4.0‰ , respectively), with only a modest drop in goodness of fit. Enrichment was less pronounced for night transects than on the preceding day (by 2 and 1.5‰ in

October and November 2009, respectively; Figure 3b); concordance between fractionation factors for samples collected in the day and those in the night one month apart is particularly striking. Comparison of day and night transects suggests that fractionation due to assimilation was, unexpectedly, higher than denitrification. Assuming fractionation and removal due to denitrification were constant, assimilation was estimated to be 5.8 and 7.7% of the total N flux for October and November transects, respectively. By comparing isotope values at the most downstream location between day and night transects, we estimated a day-night difference in $\delta^{15}\text{N}$ of 0.75 and 0.56‰ for October and November, respectively. Assuming this difference was due to the fractional effects of assimilation, and given the observed additional nitrate removal during the day, we estimated assimilatory fractionation ($^{15}\epsilon_A$) to be -9.38 and -9.33‰ , markedly higher than for denitrification (-3.09 and -3.80‰ for October and November; Figure 3b).

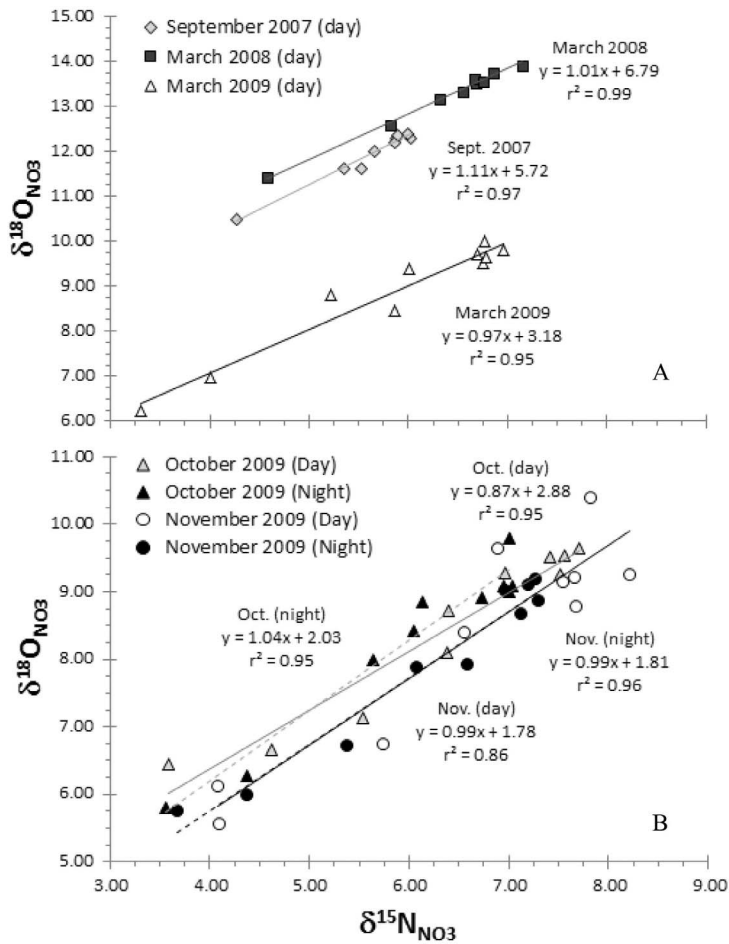


Figure 4. Dual isotope coupling (slope of $\delta^{15}\text{N}$ versus $\delta^{18}\text{O}$) from longitudinal transects (five day, solid lines, and two night, dashed lines) reveals a slope consistently near 1.0 despite mass removal dominated by denitrification.

[26] Observed patterns of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ did not support the predicted 1:2 coupling observed in other rivers with denitrification, despite the predominance of denitrification as the mechanism of N removal in the Ichetucknee [Heffernan *et al.*, 2010; Heffernan and Cohen, 2010]. Isotopic coupling, the slope of the line relating $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$, was indistinguishable from 1 for all longitudinal transects, including those done predawn in October and November 2009 (Figure 4). There was evidence of variation in the intercept values among sampling periods, with March 2008 and September 2007 exhibiting higher $\delta^{18}\text{O}$ values than other sampling events. These elevated $\delta^{18}\text{O}$ values are consistent with monthly spring vent sampling data that suggest higher isotope values during that period. The cause of variation in $\delta^{18}\text{O}$ independent of variation in $\delta^{15}\text{N}$ in vent water is unknown, but may reflect changing nitrate source loading, particularly given that the two periods of high $\delta^{18}\text{O}$ were also the periods of highest discharge (Figure 2a).

[27] Temporal variation in longitudinal fractionation varied with environmental drivers. As shown above, there was evidence of a time-of-day effect (Figure 5a) with greater fractionation during the day when both assimilation and denitrification are acting than at night when only

denitrification is occurring. We observed a strong association between the enrichment factor and flow-weighted nitrate inputs (Figure 5b) suggesting that as concentration increases, fractionation decreases; note, however, that the range of flow-weighted concentrations was small (0.56 to 0.61 mg L⁻¹) and was nearly perfectly correlated with total springs discharge ($r = +0.96$, $p < 0.01$). Hydrologic conditions also appear to control fractionation (Figure 5c), with increasing springs discharge negatively associated with enrichment (i.e., higher fractionation under conditions of lower flow). At the same time, fractionation increased as proportional water loss through the lower reach decreased (from less than 10% loss to roughly a 5% gain). Finally, fractionation decreased as total N removal on a mass basis increased (Figure 5d).

[28] Hourly sampling at the most downstream location revealed significant diel variation in isotope abundances that was approximately out of phase with diel variation in nitrate (Figure 6). Diel variation in $[\text{NO}_3\text{-N}]$ yielded estimates [Heffernan and Cohen, 2010] of autotrophic assimilation of 0.14 and 0.09 g N m⁻² d⁻¹ in March (Figure 6a) and November (Figure 6b), respectively. This estimate of assimilation was subtracted from total N removal, estimated by difference between hourly downstream concentrations

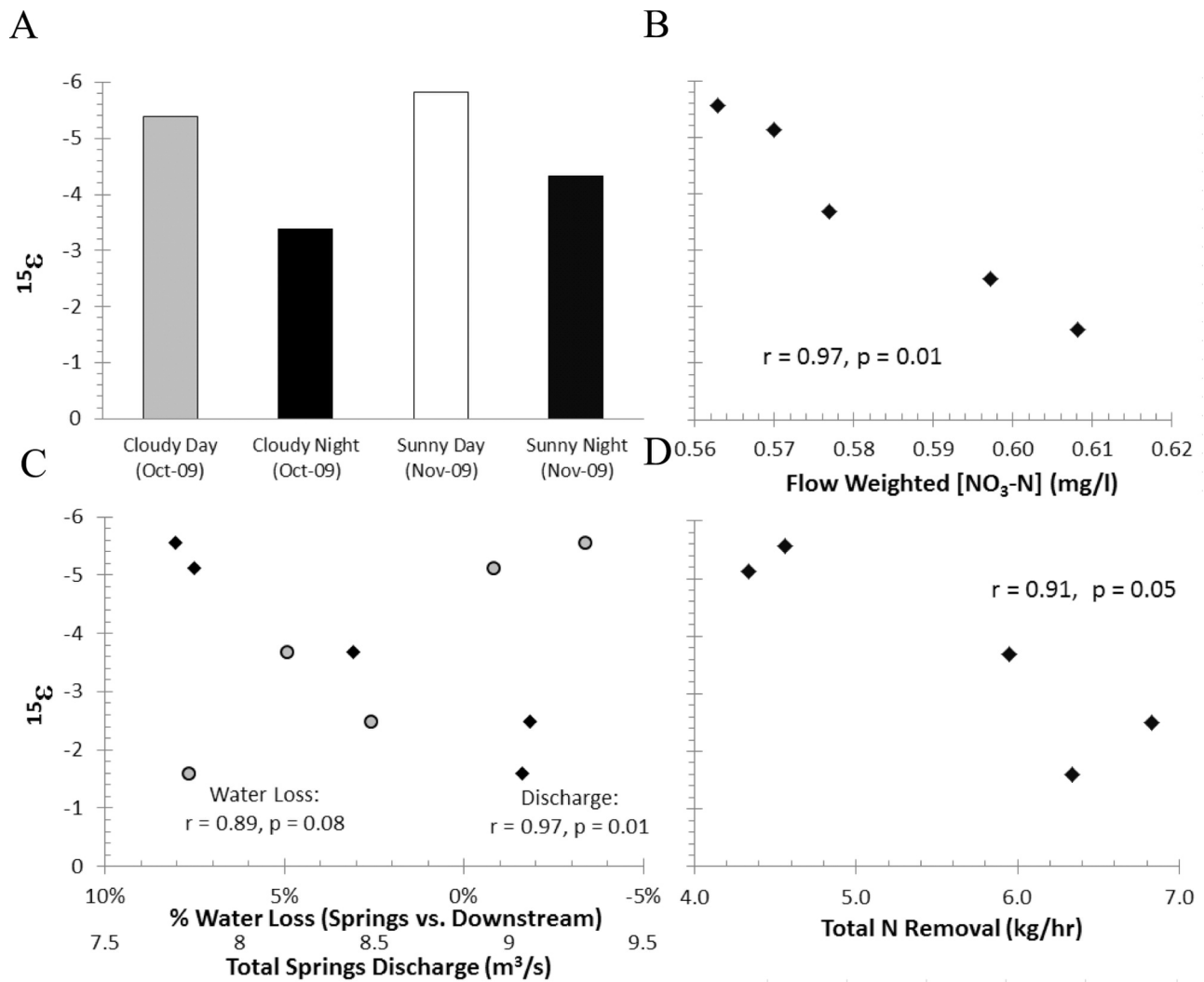


Figure 5. Controls on longitudinal fractionation including (a) time of day, (b) flow-weighted springs $\text{NO}_3\text{-N}$ inputs, (c) combined spring discharge (black diamonds) and fractional water loss (lower reach, gray circles), and (d) total riverine N removal. Effects in Figures 5b, 5c, and 5d are for daytime transects only.

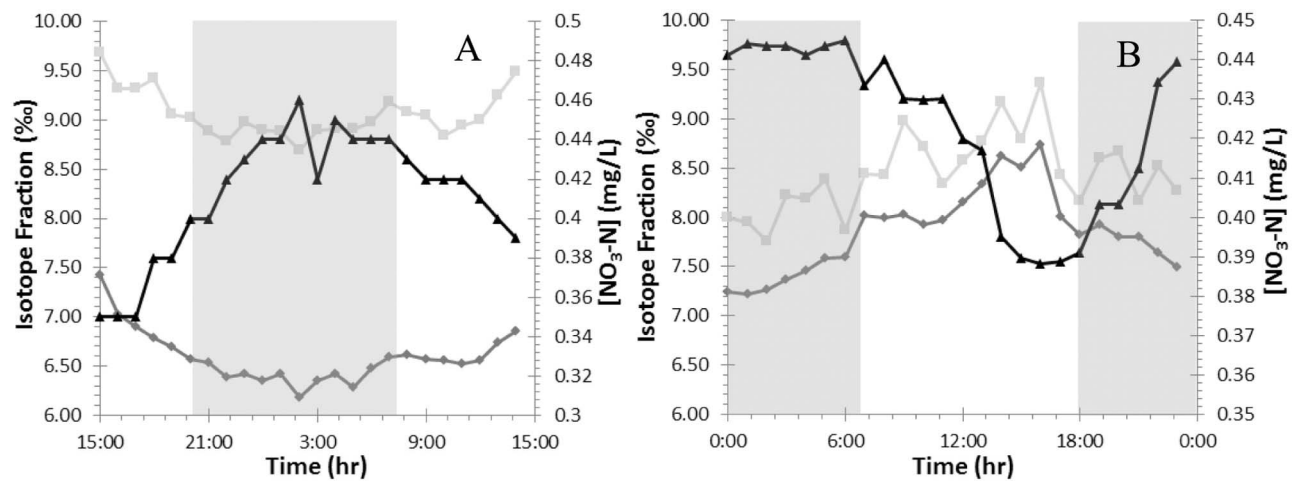


Figure 6. Diel variation in $[\text{NO}_3\text{-N}]$ (black line), $\delta^{15}\text{N}$ (dark gray line), and $\delta^{18}\text{O}$ (light gray line) for (a) March and (b) November 2009. Shaded areas denote nighttime.

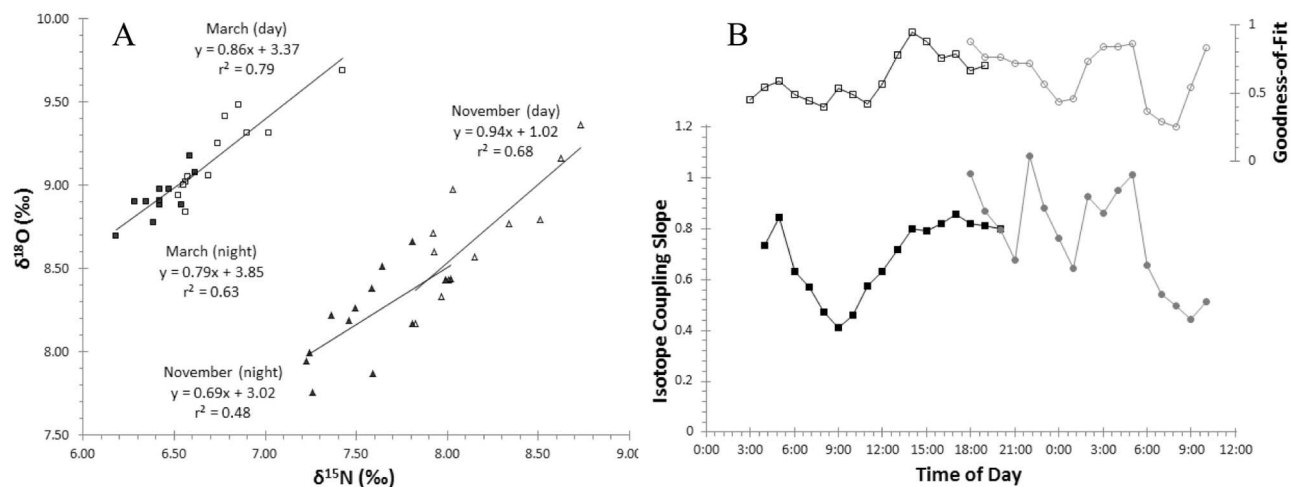


Figure 7. (a) Isotope coupling for March and November 2009, partitioned by day and night reveals slopes significantly below 1.0 ($p = 0.03$) for March but not November ($p = 0.12$); slopes were not significantly different between day and night on either date. (b) Eight hour moving window analysis of isotope coupling and goodness of fit for March (circles) and November (squares) 2009 suggests strong and consistent 1:1 coupling at all times except early morning.

and the flow-weighted input concentrations, to yield denitrification estimates of 0.61 and 0.42 $\text{g N m}^{-2} \text{d}^{-1}$ in March and November, respectively. Values of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ at the downstream location were always higher than the flux-weighted springs inputs ($D_{\text{FWA},j}$ at the most downstream station was 5.89 and 6.17‰ for $\delta^{15}\text{N}$, and 8.37 and 7.84‰ for $\delta^{18}\text{O}$ in March and November, respectively), but values were markedly higher during the day when both assimilation and denitrification were occurring. Diel patterns in $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ appear synchronous (Figure 6), but with lower temporal autocorrelation in the $\delta^{18}\text{O}$ signal.

[29] As in the longitudinal transects, diel patterns of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ did not conform to predicted 1:2 relationships. While dual isotope coupling (slope of $\delta^{15}\text{N}$ versus $\delta^{18}\text{O}$) was modestly below 1:1, this difference was statistically significant only for the March sampling ($p = 0.03$, Figure 7a). There was some evidence that nighttime slopes were shallower than daytime slopes, consistent with movement toward 1:2 coupling, but this difference was also not statistically significant. The strength of isotope coupling (goodness of fit, r^2) was also stronger in the day. We note that parsing isotope values and coupling based on day and night defined by sunrise and sunset may be confounded by the hydraulic residence time in the river ($\sim 6\text{--}8$ h [Hensley, 2010]). An 8 h moving window analysis of isotope coupling revealed greater variation in slopes (0.4 to 1.1 in March, 0.4 to 0.9 in November), with evidence of lower slopes in the early morning ($\sim 5:00$ A.M. to 10:00 A.M. EST), accompanied by evidence of decoupling (low goodness-of-fit r^2 values) (Figure 7b) at the night-to-day transition. Evidence for strengthened daytime coupling is present in the November data (squares), but not as clearly for the March data (circles). However, because each slope is derived from only 8 measurements, none of the values were significantly different from 1:1.

[30] The temporal dynamics of isotope fractionation reinforced the unexpected negative association between nitrate removal and $\delta^{15}\text{N}$, and also revealed hysteretic behavior.

The negative regression slope over time between $[\text{NO}_3\text{-N}]$ and isotope composition was significant in both March ($\delta^{15}\text{N} = 9.2 - 6.2 \cdot [\text{NO}_3\text{-N}]$; $r^2 = 0.76$; $p < 0.001$) and November ($\delta^{15}\text{N} = 13.2 - 12.3 \cdot [\text{NO}_3\text{-N}]$; $r^2 = 0.36$; $p = 0.002$), with evidence of significant counter clockwise hysteresis (Figures 8a and 8c). An estimate of total fractionation obtained hourly using a two-point removal curve (between the springs and the downstream observations) indicated low and effectively constant fractionation throughout the day in March 2009 (black circles; Figure 8b), and much higher fractionation with significant diel variation in November 2009 (black circles; Figure 8d); note a $\sim 4\text{--}6$ h river residence time. Imputing fractionation due to assimilation based on assumptions of (1) constant denitrification (i.e., diel variation in concentration was due to temporal variation in assimilation), (2) constant denitrification fractionation, and (3) zero assimilation at night yielded highly variable estimates that suggest strong diel variation ranging from -1.6 to -11.9% in March 2009 (white squares, Figure 8b) and from -2.8 to -23.4% in November 2009 (white squares, Figure 8d).

4. Discussion

[31] Unusually consistent boundary inputs (flow, chemistry, temperature), high levels of primary production, and well constrained nutrient and water mass balances make the Ichetucknee River a useful model system for investigating longitudinal and diel nitrate isotope dynamics. Other rivers where longitudinal dual isotope measurements have been obtained [Battaglin *et al.*, 2001; Ruehl *et al.*, 2007; Pellerin *et al.*, 2009; Deutsch *et al.*, 2009; Miyajima *et al.*, 2009; Chen *et al.*, 2009] are generally subject to greater variation in source and internal processing due to weather, terrestrial phenology, and landscape and river management, making inferences more complex. In addition to low variation in source chemistry, and discharge patterns, estimates of N removal rates (0.58 to 1.11 $\text{g N m}^{-2} \text{d}^{-1}$, or 27–35% of N inputs) and mechanisms (i.e., 20% assimilation, 80%

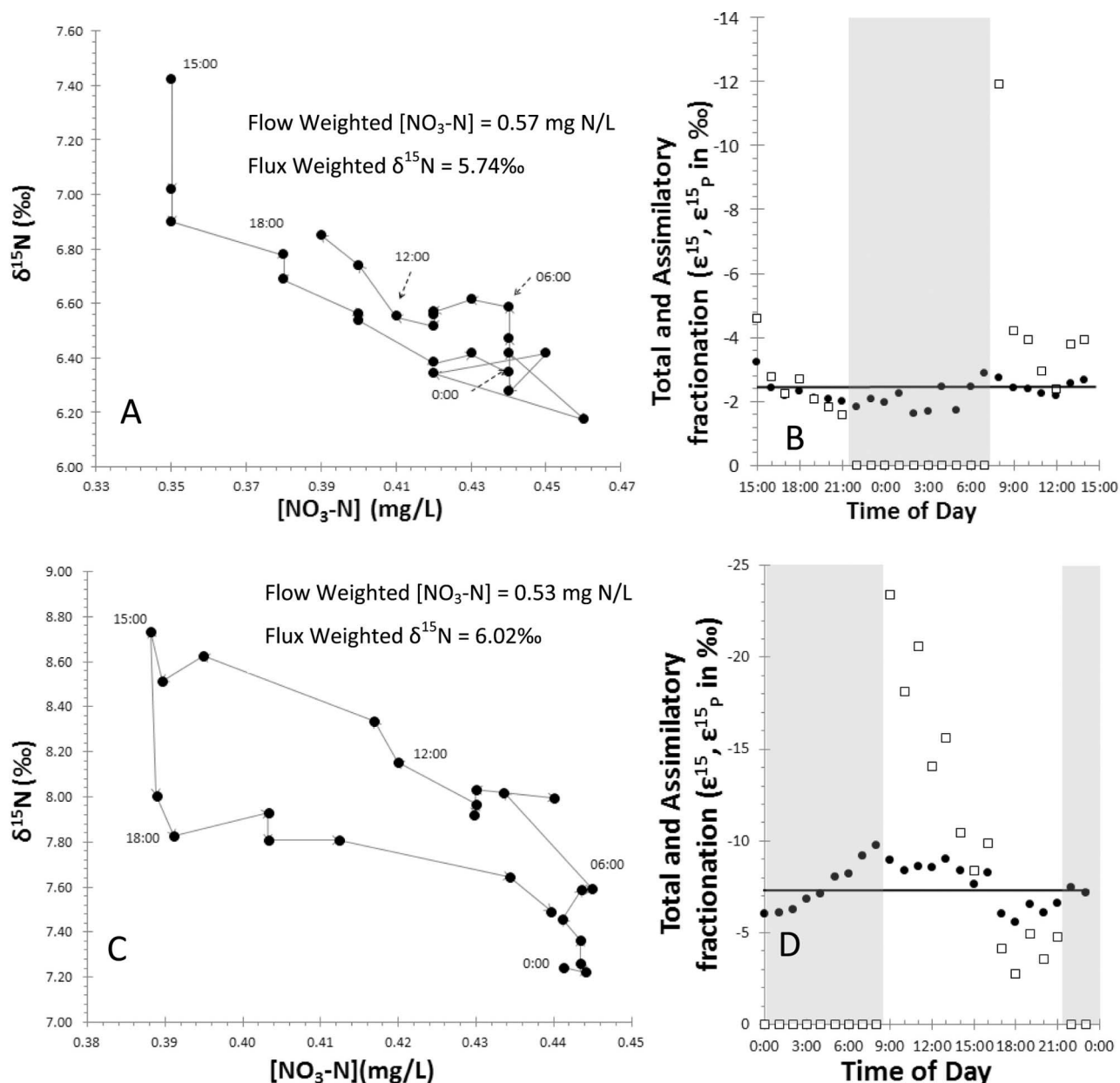


Figure 8. Diel variation in isotope values and $[\text{NO}_3\text{-N}]$ for (a) March and (c) November 2009. Springs inputs (flow-weighted $[\text{NO}_3\text{-N}]$ and flux-weighted $\delta^{15}\text{N}$) are shown for each date. Total fractionation (black dots, from (b) March and (d) November 2009) at night (gray areas) was used to estimate denitrification fractionation (horizontal black lines). Assuming constant denitrification fractionation implies significant variation in assimilatory fractionation (white squares) that drops over the course of the day.

denitrification,) align with previously published estimates [Heffernan *et al.*, 2010] and with other studies in systems with dense benthic macrophyte cover [Kreiling *et al.*, 2011], suggesting that internal variation in the relative importance of total N removal pathways is also low. As such, the complex isotope patterns we observed in the absence of hydrologic and source variation, suggest caution when inferring process from natural isotope abundance signals.

4.1. Longitudinal N Isotope Variation

[32] Isotopic fractionation was observed for all longitudinal transects, regardless of time of day or season, but the

apparent controls are different than has been observed in other rivers. Specifically, fractionation was significantly correlated with hydrologic drivers (e.g., total flow and longitudinal river losses; Figure 5c), flow-weighted input nitrate concentrations (Figure 5b) and total N removal (Figure 5d) in the Ichetucknee. However, the absence of obvious mechanisms to explain these correlations, and weak statistical power mean that further replication is required to understand biophysical controls on fractionation.

[33] Other studies [De Brabandere *et al.*, 2007; Ruehl *et al.*, 2007; Chen *et al.*, 2009] have observed greater temporal variation in riverine fractionation, either in response to

hydrologic control or season. Our results run counter to the expectations that a) increasing discharge would increase enrichment factors due to greater contact with reactive riparian sediments and/or increased hyporheic exchange as well as increased nitrate availability, and b) that higher N concentrations would lead to increased fractionation due to greater availability. Both flow and the flow-weighted input $[\text{NO}_3\text{-N}]$ were negatively associated with fractionation, though the modest range of input concentrations and the extremely strong association ($r = +0.96$, $p < 0.01$) between input concentrations and discharge, suggest that the latter correlation (fractionation versus $[\text{NO}_3\text{-N}]$) may be spurious. *Heffernan et al.* [2010] report significant positive covariance between N removal and discharge, a finding confirmed in our data set ($r = +0.97$, $p = 0.007$).

[34] One explanation for the strong discharge-enrichment association is that the relative contribution of diffuse inputs is a function of discharge. *de Montety et al.* [2011] report a chloride budget for the Ichetucknee that cannot close without invoking a lateral contribution of $1.3 \text{ m}^3/\text{s}$ of water chemically similar to Mill Pond (the most downstream spring discharging between stations 5 and 6; Figure 1). Notably, this water is generally enriched in ^{15}N and ^{18}O . If, during periods of low flow, this unaccounted for source is of greater fractional significance, the longitudinal pattern in isotope values would appear similar to fractionation. With data obtained here, we cannot reject this outright, but we note two aspects of the longitudinal data that limit the likelihood of this explanation. First, a spring's chemical characteristics are relatively constant and spatially discrete (i.e., each spring has unique chemistry), implying that the additional water similar to Mill Pond would be discharged in approximately the same location. There is no evidence from the longitudinal profiles of a consistent isotopic discontinuity that would support a relatively large unaccounted for source of water, particularly during the latter transects when the $\delta^{15}\text{N}$ values for Mill Pond were very high; we note evidence in March 2009, and November 2009 of a spike in ^{15}N near Mill Pond, but enrichment factors estimated without those points are identical to those with them. Second, the fractional water contribution of Mill Pond, flow of which should covary with the unaccounted for source of water, is constant ($\sim 8.3\%$) and unassociated with total discharge or flow-weighted input $[\text{NO}_3\text{-N}]$. We conclude that even if this unaccounted for source of water is present, its effects on spatial and temporal patterns of isotopic composition are likely to be minimal. We discuss a more parsimonious alternative for these and other observations further below.

4.2. Diel N Isotope Variation

[35] Although both ^{15}N and ^{18}O exhibited consistent diel variation (Figure 6), fractionation of ^{15}N exhibited diel variation only in one of the two diel deployments (November 2009; Figure 8d). Moreover, the direction of diel isotope variation, which was roughly out of phase with diel nitrate variation, was contrary to our expectations. We expected a higher enrichment factor due to denitrification than for assimilation, and thus a decrease in the combined enrichment factor during the day, when N is removed by both assimilation and denitrification. Instead, we observed greater daytime enrichment factors, implying assimilatory fractionation that is higher than denitrification. The magnitude of

daytime isotopic enrichment over nighttime levels indicates assimilatory fractionation in excess of -6% . This value is substantially larger than previous studies in a similar spring-fed river [*De Brabandere et al.*, 2007] where modest assimilatory fractionation (~ -2 to -3%) was inferred based on differences between water column nitrate and tissue N isotope ratios. Observed enrichment factors were also at the high end of the range reported for marine phytoplankton (-2.2 to -6.2% [*Needoba et al.*, 2003]) and benthic algae in springs (-1 to -6% [*Albertin et al.*, 2012]). Further experimental work to constrain the timing and magnitude of plant N assimilation and fractionation is clearly needed, particularly for macrophytes and epiphytes that dominate production in these systems [*Odum*, 1957; *Duarte and Canfield*, 1990].

[36] Total fractionation has been used to back-calculate assimilatory fractionation based on the assumption that fractionation due to denitrification is constant [*Dhondt et al.*, 2003]. Our findings challenge this assumption of diurnally constant denitrification rates and fractionation, at least in the Ichetucknee river. As discussed above, literature evidence suggests assimilatory fractionation is generally small, and we know of no studies that report significant diel variation therein. Assuming a constant rate and isotope effect of denitrification requires invoking untenable daytime variation in plant fractionation, ranging from highly discriminating in early morning (-11.9 and -23.4% in March and November 2009, respectively) to weakly discriminating in late afternoon (-1.6 and -2.8% ; Figures 8b and 8d).

[37] One intriguing observation is counterclockwise hysteresis in the relationship between nitrate concentration and isotope ratios ($\delta^{15}\text{N}_{\text{NO}_3}$), with evidence, particularly from the November 2009 sampling (Figure 8c), of 4 diel stages: (1) early morning increases in $\delta^{15}\text{N}_{\text{NO}_3}$ without a commensurate change in nitrate removal, (2) a rapid decline in nitrate through midday with modest isotopic effects, (3) a late afternoon decline in $\delta^{15}\text{N}_{\text{NO}_3}$, again without a change in nitrate, and (4) a nighttime increase in nitrate concentration without an isotopic effect. While the evidence for this pattern is weaker in the March 2009 sampling (Figure 8a), the timing and topology of the pattern is still evident. We suggest two possibilities. The first is that fractionation due to assimilatory removal, which drives diel nitrate variation, is highly variable, as discussed above; the magnitude of that variation is depicted in Figures 8b and 8d. We tentatively reject that explanation, based mostly on the unprecedented magnitude of the diel variation in fractionation that would be implied. A second explanation is that diel variation in the magnitude and/or fractionation due to denitrification is larger than assumed, and that variation lags variation in nitrate.

[38] Previous studies [*Heffernan and Cohen*, 2010] of high-frequency nitrate dynamics in the Ichetucknee River have inferred strong day-to-day coupling between primary production and denitrification, in which increased availability of labile organic matter following days with high GPP may cause greater oxygen reduction in the sediments, thereby enhancing denitrification. In this study, diel isotope variation was slightly higher on and after a relatively productive (i.e., sunny) November day ($\sim 1.7\%$, Figure 6b), than on and after a highly overcast March day earlier in the year ($\sim 0.7\%$; Figure 6a), which would be expected if

autotrophic activity was driving fine-scale variation in denitrification rate and/or fractionation. We note that these patterns in diel $\delta^{15}\text{N}$ amplitude occurred despite cumulative nitrogen removal (3.7 versus 5.4 kg N/h) and diel nitrate variation (0.05 versus 0.11 mg L⁻¹) that corresponded to previously documented seasonal patterns [Heffernan and Cohen, 2010].

4.3. Diel and Longitudinal Dual Isotope Coupling

[39] We observed 1:1 dual isotope coupling in both longitudinal and diel sampling, which is inconsistent with the 1:2 coupling expected for a system where N removal is dominated by denitrification [Lehmann et al., 2003]. The theoretical predictions of 1:2 coupling from Lehmann et al. [2003] are well validated in the groundwater literature [Böttcher et al., 1990; Aravena and Robertson, 1998; Mariotti et al., 1988]. Moreover, several riverine studies [Battaglin et al., 2001; Ruehl et al., 2007; Chen et al., 2009] have reported evidence of 1:2 isotope coupling due to denitrification. In contrast, however, laboratory measurements of denitrification by respiratory denitrifiers support 1:1 coupling under both fresh and saltwater conditions [Granger et al., 2008]. Deutsch et al. [2009] observed 1:1 coupling in the Elbe River, but interpreted this to indicate dominance of N removal by assimilation despite the likely transient nature of this removal as assimilated N is remineralized. Our data, combined with the laboratory culture findings of Granger et al. [2008] suggest that process inference from the slope of the isotope coupling line may be confounded, at least under the hydraulic and biogeochemical conditions in the Ichetucknee River.

[40] Systematic diel variation in dual nitrate isotope fractions has not, to our knowledge, previously been described, though studies in other rivers have observed large though generally unpatterned variation [Pellerin et al., 2009]. Piecewise measurements partitioned by day and night, defined by sunrise and sunset on each day, suggest that 1:1 coupling is ubiquitous in the Ichetucknee River. Modest departures from 1:1 slopes at night were not significant, but an 8 h moving window analysis revealed a short period in the early morning for both 24 h sampling events when the slope dropped considerably. Notably, however, this drop occurred at the same time as strong declines in goodness of fit (r^2) and temporal autocorrelation, meaning that none of the slopes could be distinguished from 1:1. In this river, decoupling cannot plausibly be explained as a change in source because of the extremely stable input chemistry and absence of additional tributaries. One potential explanation may be that the early morning period is naturally one of reduced isotope variation, and the signal of decoupling (i.e., reduced goodness of fit) is due to increased importance of measurement error. We note the temporal variation is larger for $\delta^{15}\text{N}$ than for $\delta^{18}\text{O}$ (Figure 6), in line with observed analytical precision (0.2 and 0.6‰ for $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$, respectively).

[41] Nitrification is another mechanism that could induce dual nitrate isotope decoupling because O and N are derived from different sources [Sebilo et al., 2006; Wankel et al., 2007]. The ^{15}N of nitrification-derived nitrate originates in ammonium, with modest fractionation, but the ^{18}O comes from either dissolved oxygen ($\delta^{18}\text{O}$ ranging from -24‰ to -12‰ with diel variation in primary production) or water

($\delta^{18}\text{O}$ between -4 and 0‰) (one third and two thirds, respectively [Mayer et al., 2001]); Sebilo et al. [2006] report $\delta^{18}\text{O}_{\text{NO}_3}$ after nitrification of approximately -3‰. In the Ichetucknee, where ammonium accumulation in the water column is negligible [Heffernan et al., 2010] and net assimilation is assumed slightly positive (i.e., uptake > mineralization) because of longitudinal accumulation of particulate OM, the isotopic effect of nitrification on ^{15}N of nitrate is likely neutral because the process proceeds to completion. Ambient values of $\delta^{18}\text{O}_{\text{NO}_3}$ remain between 8 and 10‰ in the Ichetucknee at all times (Figure 6), suggesting that systematic lightening $\delta^{18}\text{O}_{\text{NO}_3}$ due to nitrification is not occurring. Moreover, nitrification is expected to be enhanced during the day when pH, DO and temperature are high [Warwick, 1986; Rysgaard et al., 1994], but observed decoupling occurs when these are at their lowest values (Figure 7b).

4.4. Diffusion Controls on Denitrification and Fractionation

[42] The most parsimonious explanation for the clear divergence between expectations and observations (specifically low fractionation overall, implied diel variation in assimilatory fractionation, consistent 1:1 dual isotope coupling, diel hysteresis) is that the dominant removal process, denitrification, is limited by benthic diffusion of nitrate, the rate of which varies with ecosystem oxygen production [Rysgaard et al., 1994; Harrison et al., 2005], and may therefore not be constant [Christensen et al., 1990].

[43] Under conditions of N-sufficiency (i.e., denitrification limited by something other than nitrate) denitrification exhibits strong fractionation. Sebilo et al. [2003] showed riparian denitrification exhibited high rates of fractionation ($^{15}\epsilon = -18\text{‰}$), consistent with observations from groundwater systems [e.g., Böttcher et al., 1990; Aravena and Robertson, 1998; Fukada et al., 2003] and laboratory culture [Granger et al., 2008]. Well mixed conditions in each of these settings preclude nitrate limitation, leading to active isotope fractionation. In contrast, fractionation in river and lake sediments, where nitrate enters primarily via diffusion [Christensen et al., 1990; Rysgaard et al., 1994], exhibited much lower fractionation rates ($^{15}\epsilon = -4\text{‰}$) [Sebilo et al., 2003]. Similarly low fractionation has been observed in wetland settings where advective nitrate delivery is expected to be low [Lund et al., 1999; Søvik and Mørkved, 2008]. In the Ichetucknee River, low sediment hydraulic conductivity (<5 m/d [Hensley, 2010]) and weak hydraulic gradients (generally in the direction of river gains) limit hyporheic water exchange due to advection. As such, nitrate delivery to the anaerobic sediments where denitrification occurs must therefore be regulated by diffusion.

[44] If diffusion is the rate limiting step for denitrification, overall fractionation would be small because nitrate delivered to the active removal zone is processed in its entirety. Moreover, the flux would presumably respond to the direction and magnitude of the hydraulic gradient. During high-discharge periods, which in this river occur in response to increased groundwater elevations not surface drainage, the hydraulic gradient is more strongly toward the river (i.e., gaining conditions), which would in turn reduce diffusion into the sediments and lower fractionation, as observed (Figure 5c); in contrast, periods of high stage on the

Ichetucknee are driven by downstream flooding (via back-water effects) which are rare, but could reverse the hydraulic gradient. We note that other rivers would likely be reversed to typical conditions in the Ichetucknee, with periods of high discharge corresponding with high infiltrating hydraulic gradients forcing water into the sediments.

[45] Invoking diffusion limitation as the primary control on denitrification is also consistent with a negative association between fractionation and total N removal. As conditions become more favorable for denitrification (i.e., more available organic carbon as substrate [Heffernan and Cohen, 2010]), that process proceeds to completion at those sites where the combination of conditions (i.e., low redox, bio-available organic matter and consistent nitrate delivery) are met, minimizing fractionation [Houlton et al., 2006]. The association between flow-weighted input concentrations and fractionation (Figure 5b) is difficult to explain, potentially indicating a spurious relationship driven by the strong effect of discharge on both removal and N concentration.

[46] Considering denitrification as limited by benthic diffusion rates may also account for the observed 1:1 dual isotope coupling. Specifically, while denitrification fractionation under conditions of saturated N supply may create 1:2 coupling, fractionation under N-limited conditions may be different. N isotope fractionation is reduced substantially when nitrate is supplied to denitrifiers via diffusion [Sebilo et al., 2003], but the simultaneous effects of diffusion limitation on $\delta^{18}\text{O}$ fractionation remains unknown. If diffusion limitation exerts equal fractionation on both N and O isotopes, and renders the process nitrate limited, the fine-grained sediments and low hydraulic gradients in the Ichetucknee would yield different coupling than has been observed in coarser grained or higher-gradient systems.

[47] In addition to helping explain weak fractionation and 1:1 coupling, diffusion control on denitrification is also consistent with the observed diel signals. To reiterate, the assumption of constant denitrification magnitude and fractionation forces the conclusion of large diel variation in assimilation fractionation. If we reject the implied magnitude and variation in assimilatory fractionation as implausible, the remaining explanation for observed diel variation in isotope values is that the magnitude of and/or fractionation due to denitrification is not a constant. If the denitrification flux is diffusion limited, diel variation in diffusion length would also be expected; during the day, DO produced in the water column diffuses more strongly into the benthic sediments, which in turn increases the effective depth to which nitrate must diffuse to reach favorable redox conditions for denitrification [Christensen et al., 1990; Harrison et al., 2005]. While diffusion-limited denitrification may be slower in magnitude [Christensen et al., 1990] and weakly fractionating overall [Sebilo et al., 2003], the kinetics of fractionation at the ecosystem level likely vary in response to changing diffusion length. Under conditions of low river DO, greater fractionation would be expected because an increasing number of sites in the river have short-enough diffusion lengths to make denitrification N limited. In contrast, under high DO conditions, diffusion lengths increase, and diffusion limitation becomes relatively more prevalent, lowering fractionation.

[48] If diel variation in diffusion length is occurring, the observation that isotope changes lag somewhat behind

changes in nitrate (which are coincident with changes in dissolved oxygen) would also be expected. In other words, the observation of diel hysteresis may result from a lag between changes in water column conditions and resulting changes in sediment conditions. During the predawn to early morning phase of the diel cycle, nitrate concentrations remain constant, but fractionation increases (i.e., isotope values are generally increasing). We propose that this idea is consistent with diffusion limited denitrification where several hours of reduced DO concentrations in the water column limit the depth to which nitrate must diffuse to reach favorable redox conditions [Christensen et al., 1990], in turn increasing fractionation. Likewise, in the late afternoon to early evening, nitrate varies little, but fractionation decreases, consistent with greater diffusion limitation in response to increased water column DO concentrations (peak at 3:00 P.M. EST), but where that inhibition of denitrification is slightly lagging (by ~ 3 h). Further testing in both controlled conditions and other rivers is needed to determine if this diel variation in diffusion length is occurring, whether it can plausibly explain the diel isotope variation, whether the effect is general to rivers with low hydraulic conductivity sediments.

[49] The implication of potentially substantial diel variation in denitrification is broadly relevant. First, our inference of N assimilation, based on the assumption that autotrophic uptake is the sole source of diel nitrate variation, would consequently be an underestimate, and methods for decomposing the diel signal into assimilatory and dissimilatory components would be necessary. More importantly, however, evidence counter to the assumption of diurnally constant denitrification influences models of river network N removal, which would be an underestimate if predicated only on daytime sampling, when denitrification would be expected to be reduced. Mulholland et al. [2008] report no variation in denitrification uptake velocity between midnight and noon across 72 creeks in 8 biomes, though sufficiency of a two-point contrast, particularly at those times of day, is unknown. In contrast, several studies have reported large diel changes in N processes associated with redox variation [Warwick, 1986; Christensen et al., 1990; Valett, 1993; Harrison et al., 2005], including patterns reverse to those observed here where denitrification is enhanced during the day possibly due to coupling with nitrification [Laursen and Seitzinger, 2004], providing ample precedent for diel denitrification variation inferred here.

5. Conclusions

[50] Overall, our results reveal several novel behaviors of natural isotope abundances in a highly stable river. These include hysteresis between nitrate concentrations and isotope abundances, and diel variation in fractionation, which we interpret as evidence of diel variation in denitrification induced by diffusion-mediated N limitation of that process. These inferences are also consistent with other observations, including low overall fractionation rates and temporal invariance of both fractionation and ^{15}N : ^{18}O coupling despite dramatic diel variation in the magnitude of assimilation. Despite the unusual stability of upstream boundary conditions, these same observations rendered our original objective of using diel and longitudinal dual isotope

variation to determine the magnitude of denitrification and assimilation impossible. The generality of this diffusion limitation mechanism for environmental isotope dynamics (e.g., in low relief or gaining rivers where hyporheic exchange is controlled by diffusion), the observed controls on fractionation, and the inference of diel hysteresis and implications for variation in dissimilatory N removal are findings that merit further scrutiny in this and other rivers. Overall, our results suggest, on one hand, that inference of N removal processes from fractionation and isotope coupling should be done with caution, as these indicators may not hold in all settings. On the other hand, in conjunction with other observations (i.e., high-frequency solute chemistry), dual isotopes have the potential to provide unique means for testing hypotheses about coupling of metabolism and N cycling and interactions among N transformations.

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