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Increased soil stable nitrogen isotopic ratio following phosphorus enrichment: historical patterns and tests of two hypotheses in a phosphorus-limited wetland

P. W. Inglett · K. R. Reddy · S. Newman · B. Lorenzen

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Abstract We used a P enrichment gradient in the Everglades to investigate patterns of the stable N isotopic ratio (δ^{15}N) in peat profiles as an indicator of historic eutrophication of this wetland. We also tested two hypotheses to explain the effects of P on increased δ^{15}N of organic matter including: (1) increased N mineralization/N loss, and (2) reduced isotopic discrimination during macrophyte N uptake. Spatial patterns of δ^{15}N in surface litter and soil (0–10 cm) mimic those of the aboveground macrophytes (*Typha domingensis* Pers. and *Cladium jamaicense* Crantz). Peat profiles also show increased δ^{15}N in the peat accumulated in areas near the historic P discharges since the early 1960s. The increased δ^{15}N of bulk peat correlated well with both measured increases in soil total P and the historical beginning of nutrient discharges into this wetland. In 15-day bottle incubations of soil, added P had no effect on the δ^{15}N of NH_4^+ and significantly increased the δ^{15}N of water-extractable organic N. Measurements of surface soils collected during a field mesocosm experiment also revealed no significant effect of P on δ^{15}N even after 5 years of P addition. In contrast, δ^{15}N of leaf and root tissues of hydroponically grown *Typha* and *Cladium* were shown to increase up to 12‰ when grown at elevated levels of P and fixed levels of N (as NH_4^+). The magnitude of changes in δ^{15}N resulting from altered discrimination during N uptake is significant compared with other mechanisms affecting plant δ^{15}N, and suggests that this may be the dominant mechanism affecting δ^{15}N of organic matter following P enrichment. The results of this study have implications for the interpretation of δ^{15}N as an indicator of shifts in relative N limitation in wetland ecosystems, and also stress the importance of experimental validation in interpreting δ^{15}N patterns.

Keywords Peat profile · Wetland eutrophication · Nitrogen isotopes · Biogeochemistry · Macrophytes

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Introduction

In addition to tracing the flow and utilization of organic matter in foodwebs, stable N isotopic ratios (δ^{15}N) have been used as an indicator of the historical nutrient status of lakes (Brenner et al. 1999), estuaries (Orem et al. 1999), and more recently, wetlands (Novak et al. 1999; Wooller et al. 2003). In these studies the δ^{15}N of sedimented/accreted organic matter, either algal-, microbial- or macrophyte-derived, is used to infer changes in nutrient conditions within a given system. Many processes are known to affect the production, composition, and fate of organic matter, and therefore, knowledge of these factors and their effects on organic matter δ^{15}N is essential for successful application of this technique in a given system.

Conceptually, the processes affecting the $\delta^{15}N$ of organic matter can be divided into two categories, namely, those determining the isotopic ratio of the living biomass, and those affecting the isotopic ratio of the detrital material during decomposition. In the case of living biomass, $\delta^{15}N$ is largely derived from the combination of $\delta^{15}N$ of the N source and the physiological mechanisms governing fractionation during plant uptake (Evans 2001; Handley and Raven 1992). In this manner, most macrophytes have $\delta^{15}N$ values below that of the available N within a system. As plant N demand increases, less isotopic discrimination during N uptake/assimilation will occur until ultimately, the plant becomes isotopically identical to its N source. This is particularly important during the uptake of NH_4^+, and as a result, macrophyte $\delta^{15}N$ is beginning to be proven useful at indicating relative N and P limitation in wetlands (Mckee et al. 2002; Clarkson et al. 2005; Inglett and Reddy 2006).

In a recent study of wetland macrophyte $\delta^{15}N$, Inglett and Reddy (2006) observed higher $\delta^{15}N$ in plants growing near the discharge of high P drainage waters in the Everglades. In peat-based wetland systems such as the Everglades, the overwhelming majority of soil N is that of plant-derived organic-N, and therefore, it is logical that the isotopic composition of the soil would reflect that of the litter materials contributed by the dominant macrophytes. However, a number of processes can alter organic matter $\delta^{15}N$ during decomposition, and thus can greatly affect interpretation of organic matter $\delta^{15}N$ patterns (discussed by Lehmann et al. 2002). Among these, immobilization can increase or decrease $\delta^{15}N$ by the incorporation of external N (Benner et al. 1991; Fogel and Tuross 1999). During mineralization, loss of high $\delta^{15}N$ compounds (e.g., amino acids) can also lower $\delta^{15}N$; however, more frequently, decomposition is thought to increase $\delta^{15}N$ of litter material through the loss of isotopically light N (Nadelhoffer and Fry 1988). Also, most mechanisms of N loss (e.g., nitrification, denitrification) are selective for ^{14}N (Bedard-Haughn et al. 2003), therefore, preferential loss of excess ^{14}N from a system over time leaves the residual N pool enriched in ^{15}N (Hogberg 1990, 1991; Martinelli et al. 1999). By this mechanism, alteration of N turnover and retention rates could potentially change the $\delta^{15}N$ of the existing soil N reservoir.

Further complicating interpretation of organic matter $\delta^{15}N$ is the fact that a major contributor to the isotopic signature of macrophytes in a peat system undoubtedly comes from N mineralized from the peat soil (Nadelhoffer et al. 1996). In this manner, the isotopic changes observed in the plants may be the direct result of isotopic changes in the soil, and vice versa. In nutrient-impacted systems, this results in a circular argument, where it is unknown which isotopic change (in the soil or the plant) precedes the other. Uncertainty in the origin of the derived $\delta^{15}N$ signature has important implications regarding the chronological accuracy of $\delta^{15}N$ changes in a peat profile. Currently, there are few datasets to refine the importance of either pre- or post-depositional processes in affecting changes to soil $\delta^{15}N$.

In order to better understand the causes of increased wetland $\delta^{15}N$, and to improve the use of $\delta^{15}N$ as a predictor of N cycle alteration, it is necessary to assess the relative contribution of pre- and post-depositional mechanisms affecting $\delta^{15}N$ in ecosystem components. For this reason, the following study was undertaken to investigate spatial and historical patterns of wetland soil $\delta^{15}N$ as they relate to macrophyte $\delta^{15}N$ within a well-studied P enrichment gradient of the Florida Everglades ecosystem. Additional experiments were then conducted to test two possible mechanisms for altering N isotopic ratios. Two approaches were taken. In the first approach, $\delta^{15}N$ was measured on samples obtained from two previous studies including: (1) a hydroponic growth study of wetland macrophytes grown at both low and high levels of P enrichment (Lorenzen et al. 2001), and (2) a long-term field P dosing experiment in a historically P-limited soil (McCormick and O'Dell 1996; Newman et al. 2004). The second approach utilized a laboratory study to attempt to directly measure the isotopic composition of N mineralized from wetland soils incubated with various levels of added P.

Materials and methods

Site description

Water conservation area 2A is a diked, 547-km^2 portion of the northern Everglades ecosystem (Fig. 1). WCA-2A is

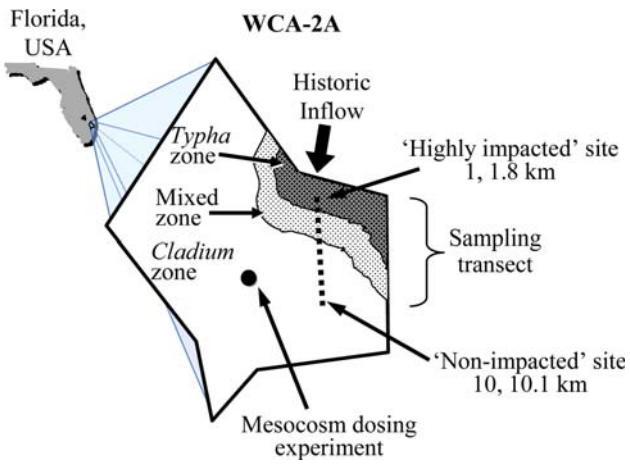

Fig. 1 Location of water conservation area 2A, the transect of stations used for spatial characterization of stable N isotopic ratios ($\delta^{15}N$), the mesocosm P-dosing experiment (McCormick et al. 1996; Newman et al. 2004), and sites of soil collection for the bottle mineralization experiment of this study

characterized as a peat-based, freshwater wetland underlain by limestone substrata. In its natural state, WCA-2A is a highly P-limited system; however, inputs of high-P agricultural drainage water along the northeastern perimeter have decreased P limitation in impacted areas. The result of the drainage discharges has been the creation of a P enrichment gradient (Koch and Reddy 1992; Reddy et al. 1998), and a shift toward greater N limitation near the inflows (McCormick et al. 1996; Inglett et al. 2004). The vegetation of WCA-2A has also shifted along the P gradient from interior marshes consisting primarily of sawgrass (*Cladium jamaicense* Crantz) to nearly monotypic stands of cattail (*Typha domingensis* Pers.) in the nutrient-affected inflow areas (Childers et al. 2003; Craft and Richardson 1997).

Spatial and historical patterns of soil $\delta^{15}N$

Spatial patterns of $\delta^{15}N$ were assessed using samples collected in September 2000 from a transect of ten sites spanning the WCA-2A nutrient gradient (Fig. 1). At each of these sites, samples of soil (0–10 cm) and surface litter were collected at random within an approximately 10-m × 10-m marsh area. Soil samples were taken using a sharpened, thin-wall aluminum tube (10 cm diameter). Samples were placed on ice and returned to the laboratory where soils were screened to remove macroorganic components (e.g., large roots). All samples were dried at 55°C and ground to pass a 2-mm mesh using a Wiley mill. Subsamples of soil and litter were then ball milled for bulk chemical and isotopic analyses.

Historical patterns of $\delta^{15}N$ were assessed in vertical peat profiles using samples of intact peat cores from a previous study of WCA-2A (Reddy et al. 1993, Fig. 1). These cores were obtained in 1991 from sites along a similar transect to that used in this current study and correspond to sites of high (1.8 km), medium (3.5 km), and low (8.3 km) levels of P enrichment. The cores were sectioned in 1-cm intervals, screened to remove large root material, dried at 70°C, and ball milled for chemical analysis. For the current study, these samples were again analyzed for total N (TN), total C (TC), and $\delta^{15}N$ in February 2002.

Effect of P addition on $\delta^{15}N$ of soil N mineralization products

P loading has been shown to increase the overall activity of soil microorganisms in P-limited Everglades soils (Amador and Jones 1993; Reddy et al. 1999). This increased activity is also believed to result in increased NH_4^+ release from Everglades soils amended with P (Newman et al. 2004; White and Reddy 2000). The determination of N isotopic composition of N mineralized from WCA-2A soils was conducted using surface soils (0–10 cm) collected in February 2003 from two sites representing the high- and low-nutrient extremes of the WCA-2A transect (sites 1 and 10, Fig. 1). Bulk soils from each site were screened to remove large root and rhizome material and homogenized. Three replicate subsamples of soil (200 g wet weight) from each site were placed into 500-ml media bottles to which was added 300 ml of distilled–deionized water containing either 0 (control), 3.5, or 7.0 mg P (added as Na_2HPO_4). The 3.5 and 7.0 mg P additions were designed to increase the soil P level of the non-impacted site 10 soils by roughly 50 and 100%, respectively. O_2 was removed from the bottle solution by bubbling with N_2 for 5 min. The closure threads were wrapped in parafilm and the bottles were incubated horizontally in the dark inside a temperature-controlled shaker for 15 days at 25°C. Bottles were shaken horizontally once daily for 1 min at 100 r.p.m.

Three replicate bottles representing each site and receiving no P addition were collected initially to characterize water-extractable soil N. The remaining 18 bottles collected after the 15-day incubation were processed in a similar manner as follows. Each bottle was shaken to create a slurry and poured into replicate 250-ml centrifuge bottles and centrifuged at 6,000 r.p.m. for 10 min. The supernatant from each bottle was filtered (Whatman no. 41) and either acidified (H_2SO_4) for determination of NH_4^+ and dissolved total Kjeldahl N (TKN) or left unacidified for determination of $\delta^{15}N$ of NH_4^+ and TN.

Long-term effect of P addition on soil $\delta^{15}N$

The effect of long-term P dosing on soil $\delta^{15}N$ was assessed using samples obtained from a 5-year P dosing experiment conducted by the South Florida Water Management District (McCormick and O'Dell 1996; Newman et al. 2004). This experiment was conducted at a non-impacted site in the interior of WCA-2A (Fig. 1). At this location, 24 individual plots (1.8 m^2) were designated and either enclosed with transparent fiberglass cylinders (1.2 m high × 1.5 m diameter) (21 plots) or left open as controls (three plots). The enclosed plots were dosed weekly with ortho-phosphate (NaH_2PO_4) at seven rates (0–12.8 g P m^{-2} $year^{-1}$) from June 1995 to April 2000. Surface soil samples (0–3 cm) were obtained periodically throughout the dosing period, dried at 85–90°C, and ground for nutrient determination. For this current analysis, only samples of the closed and open controls (0 g P added) and highest loading (12.8 g P m^{-2} $year^{-1}$) treatments were used. These archived samples were analyzed for TN, TC, and $\delta^{15}N$ in April, 2004, at the University of Florida, Gainesville, Florida.

Effect of P addition on macrophyte $\delta^{15}N$

One test of the effect of P addition on $\delta^{15}N$ of macrophytes is to compare plants grown at different levels of P, but the same level of N. It was hypothesized that with P addition, increasing N demand would decrease plant discrimination relative to the N source (as NH_4^+). In this manner, the potential for increasing plant $\delta^{15}N$ through altered N uptake would be illustrated. This effect was determined using samples obtained from an experiment by Lorenzen et al. (2001), where specimens of Everglades *Cladium* and *Typha* were grown under constant N supply at varying levels of P.

This experiment was carried out at the PhytoNutriTron hydroponic growth facility at the Department of Plant Ecology, University of Aarhus (Lorenzen et al. 1998). In this facility, plants were grown in climate-controlled chambers in solution culture maintained at P levels ranging from 10 μg P l^{-1} (control) to the highest level (500 μg P l^{-1}), while N (as NH_4^+) was supplied at a constant level (2.4 mg l^{-1}) in all treatments. Nutrient levels were continuously maintained using an automated monitoring and dosing system which ensured that concentrations were maintained within ±10% of the desired set point during experimental periods. At 0, 12 and 18 days of the experiment, from two to four plants from each replicate treatment were harvested at random and divided into the main shoot system (leaves and shoot base), rhizomes and roots. The fractions were washed in distilled water, oven-dried at 80°C, and ground for nutrient analysis. For this current study, only samples collected at 18 days from the control (10 μg P l^{-1}) and high (500 μg P l^{-1}) treatment were used. These stored samples were analyzed for TN, TC, and $\delta^{15}N$ in January 2004 at the University of Florida, Gainesville, Florida.

Chemical and isotopic analyses

NH_4^+ was measured colorimetrically on acidified extract samples using a Technicon autoanalyzer (method 350.1, USEPA 1993). TKN was measured similarly following Kjeldahl digestion (Method 352.1, USEPA 1993). Non-acidified extracts were frozen and prepared for $\delta^{15}N$ analysis of TN by lyophilizing 20-ml aliquots and analyzing the resulting powder for TN and $\delta^{15}N$. $\delta^{15}N$ of NH_4^+ was measured using an adapted version of the teflon tape diffusion method (Sorensen and Jensen 1991; Stark and Hart 1996). Briefly, this method utilized MgO (0.2 g per 70 ml solution) to convert sample NH_4^+ (>75 μg N) to NH_3 gas which was then trapped using a glass fiber filter disk (7 mm diameter, Whatman GF-D) acidified with 20 μl of 0.5 M $KHSO_4$. The acidified filter disk was protected by placing it inside a folded piece of commercially-available teflon pipe sealing tape (2.5 cm-wide × ~4 cm long). The teflon tape packets were press-sealed along the edges and then floated on the surface of the sample solution containing MgO inside in an air-tight vessel (standard 500-ml Mason jar). Jars containing packets were placed into an incubator/shaker (70 r.p.m. at 30°C) and allowed to diffuse for 6 days. The teflon tape packets were then removed from the jars, rinsed with distilled–deionized water, gently blotted, and allowed to dry overnight in a desiccator with a beaker of concentrated H_2SO_4. After drying, the packets were opened to remove the glass fiber filter disk which was then folded and wrapped in a tin capsule for TN and N isotopic analysis.

Total N, TC, and $\delta^{15}N$ ratios of soil, plant tissue, and glass fiber diffusion disks were determined using a Costech model 4010 elemental analyzer (Costech Analytical Industries, Valencia, Calif.) coupled to a Finnigan MAT Delta^{Plus}XL mass spectrometer (continuous flow isotope ratio mass spectrometry; Thermo Finnigan, San Jose, Calif.) via a Finnigan Conflo II interface. Ratios of ^{15}N:^{14}N in samples (R_{sample}) are expressed as per mil (‰) differences from the ratio of standard atmospheric N_2 (R_{std}) using delta notation (δ) as: $\delta^{15}N_{sample} = [(R_{sample}/R_{std}) - 1] \times 1{,}000$. Elemental calibration was accomplished using acetanilide (10.4% N, 71.1% C), and measurements were verified using a standard wheat flour (1.85% N, 40.2% C, $\delta^{15}N = 2.55‰$; Iso-Analytical, Cheshire, UK) and $(NH_4)_2SO_4$ (IAEA-N1, $\delta^{15}N = 0.4‰$; IAEA-N11, $\delta^{15}N = 3.5‰$). Analytical precision for standards during this period was less than ±0.3‰.

Calculation of $\delta^{15}N$ in released NH_4^+ and organic-N

Organic-N was calculated as the simple difference between TN (as TKN) and NH_4–N. During the bottle mineralization study it was assumed that there were no N losses (either through denitrification or NH_3 volatilization), therefore, net quantities of N released (as NH_4^+ and organic-N) during the bottle mineralization study were then determined by subtracting the initial amounts of NH_4-N or organic-N in water extracts from those present after the 2-week incubation. The $\delta^{15}N$ of organic-N was then calculated using values of $\delta^{15}N$ of NH_4^+ and TN, and the elemental concentrations of NH_4-N, organic-N and TKN measured in the extracts as follows:

$$(\delta^{15}N)_{Org-N} = \frac{(N \times \delta^{15}N)_{TN} - (N \times \delta^{15}N)_{NH_4^+}}{(N)_{Org-N}}$$

In this calculation, it is important to note that the $\delta^{15}N$ of neither NH_4^+ nor organic-N reflects the isotopic compositions of actual compounds released or mineralized during the study, but reflects the net result of both mineralization, release, and immobilization processes.

Statistical analyses

All statistical tests were conducted using Statistica 6.1 (Statsoft, Tulsa, Okla.). The effect of long-term P addition on soil δ^{15}N in the mesocosm experiment was evaluated using a two-factor ANOVA with main effects of mesocosm treatment [open-control, closed-control, P-loaded (12.8 g P m^{-2} $year^{-1}$)], and time (1995, 1996, 1997, 2000). Resulting δ^{15}N of N forms mineralized in the laboratory bottle incubation was assessed using the general linear model multiple ANOVA procedure with fixed effects of soil type (site 1 and site 10), P addition level (0, 3.5, 7.0 mg P), applying the full factorial interaction. All post hoc comparisons of means for significant effects and interaction ($\alpha = 0.05$) were accomplished using Fisher's least significant difference test.

Results and discussion

Spatial and historical patterns

It has previously been demonstrated by Inglett and Reddy (2006) that plants of WCA-2A respond to the inputs of agricultural drainage waters with increasing δ^{15}N. Surface litter material and soils on the WCA-2A transect also show increased δ^{15}N (Fig. 2) with values ~4‰ higher near the inflow sources relative to the interior marsh. Values were also nearly identical for both litter and surface soil at all transect sites (Fig. 2). In peat systems, soils form through accretion of macrophyte biomass, therefore, the similarity between litter and soil is not surprising. However, this contrasts with other studies showing gradual alteration of original plant δ^{15}N during diagenesis (Fogel and Tuross 1999; Melillo et al. 1989). In this case, the similarity between the litter and soil at all WCA-2A transect sites indicates that the immobilization and mineralization processes affecting δ^{15}N are rapid and largely completed during the early phases of litter diagenesis (0.5–1 year). For this reason, the δ^{15}N of peat could serve as an accurate record of conditions at the time of litterfall, and thus, is also a sensitive temporal indicator of nutrient enrichment.

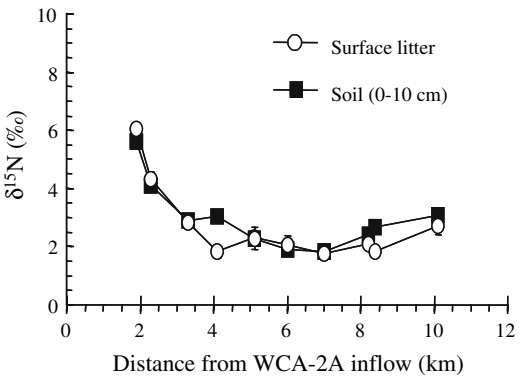

Fig. 2 Patterns of δ^{15}N of surface litter materials and soils (0–10 cm) along the nutrient gradient of water conservation area 2A

Analysis of the sectioned cores displays the peat isotopic record in WCA-2A (Fig. 3). In the case of the non-impacted core, it is clear that the peat of WCA-2A is very stable throughout the past century. In other studies of wetland peat it has been observed that there is a steady maturation of peat with increasing age, and this reflects a steady enrichment of δ^{15}N with depth in the core profile (e.g., Novak et al. 1999). Such downcore increases in δ^{15}N are presumed to result from N loss processes (N mineralization, denitrification, etc.); however, the results presented in Fig. 3, demonstrate that like the elemental C and N composition, the δ^{15}N of bulk peat N remains remarkably constant (at ~2.5‰) throughout the profile of the non-impacted WCA-2A core (8.3 km). Currently, there is no explanation for this lack of change with depth in these Everglades soils; however, the constant δ^{15}N in WCA-2A could represent a general lack of microbial activity (and N cycling) as a result of P limitation in these soils.

In contrast to the non-impacted site, the isotopic profile of the 1.9 km site clearly shows the effect of nutrient enrichment in a manner similar to the isotopic changes of the transect surface components (Fig. 3). These changes included a gradual enrichment of P beginning about 13 cm in the profile, and concurrent with the P increase, a gradual alteration of the δ^{15}N peat signature toward the surface. Dating of the core sections using the ^{137}Cs profile method (Reddy et al. 1993) demonstrated that P enrichment and the changes in δ^{15}N began to occur around or shortly after 1964. In a historical context, records indicate that discharges into WCA-2A began in the late 1950s, and 1960–1963 marked the completion of the WCA-2A inflow control structures just north of the impacted site of this study (Bartow et al. 1996; Light and Dineen 1994).

Potential mechanisms of δ^{15}N increase

Many mechanisms are known which can increase or decrease system δ^{15}N and therefore could be used to explain the downcore δ^{15}N patterns. Perhaps the most obvious of these is the presence of an isotopically enriched N source on the WCA-2A drainage water inflows. This possibility was discussed by Inglett and Reddy (2006) with the conclusion that the δ^{15}N of inflow N (4‰) was only slightly higher than that of the ecosystem components of the WCA-2A interior. For this reason, Inglett and Reddy (2006) concluded that this mechanism alone could not be used to explain the increased δ^{15}N of the live macrophytes.

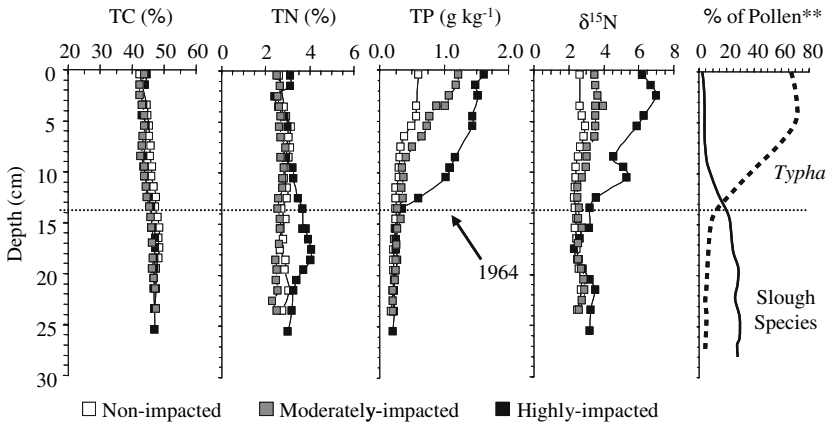

Fig. 3 Vertical patterns of C N, P, $\delta^{15}N$, and dominant pollen composition of peat soils from impacted (1.8 km from inflows), moderately impacted (3.5 km), and non-impacted (8.1 km) WCA-2A sites. The 1964 date (*arrow*) was determined using the ^{137}Cs peak method (Reddy et al. 1993) and is shown for the impacted core only. **Data for percent of pollen are summarized from Bartow et al. (1996) for a core taken at 1.4 km from the WCA-2A inflow. *TC* Total C, *TP* total P, *TN* total N

Alternatively, increased utilization of N derived from N_2 fixation (having a $\delta^{15}N = 0‰$) may explain lowered $\delta^{15}N$ values. Isotopic composition of N_2-fixing algal biomass has been shown to decrease with increases in nitrogenase activity (indicated by C_2H_2 reduction) (Inglett et al. 2004; Rejmakova et al. 2004). In the Everglades, this mechanism could be quite significant due to both an abundance of N_2-fixing cyanobacterial periphyton and overall high rates of nitrogenase activity in many of these systems (Inglett et al. 2004). Despite this potential effect, however, it has also been shown that P loading increases overall rates of nitrogenase activity in both the periphyton and detrital ecosystem components of WCA-2A (Inglett et al. 2004). Increased nitrogenase activity should result in lowered $\delta^{15}N$ in P-impacted Everglades areas, therefore it is unclear how this mechanism can be used to explain the increased $\delta^{15}N$ in P-affected WCA-2A soils.

Other mechanisms affecting $\delta^{15}N$ include the enhancement of previously discussed microbial processes (N mineralization, denitrification, etc.). In this manner, P additions may lead to increased microbial activity, N mineralization, and subsequent loss of isotopically light N from the system. Increased respiration, N mineralization, and rates of potential denitrification have been noted in Everglades soils amended with P (Amador and Jones 1993; Newman et al. 2004; White and Reddy 2000, 2003). If sufficiently increased, these processes are known to discriminate against ^{15}N, and thus, N loss following P enrichment of Everglades soils could increase the $\delta^{15}N$ of the residual soil N. A strong correlation between soil $\delta^{15}N$ and total P (TP) lends support to this hypothesis (Fig. 4), indicating that $\delta^{15}N$ becomes enriched at a rate proportional to the amount of P in the system.

Effect of P on $\delta^{15}N$ of soil N mineralization products

If NH_4- and organic-N released following P addition is sufficiently light isotopically, the residual soil N pool may become measurably enriched in ^{15}N. A direct measurement of water-extractable N predicts that available NH_4^+ present in the low P soils of WCA-2A (Table 1, site 10) maintains a $\delta^{15}N$ signature approximately 7‰ higher than the bulk soil N, while that of water-extractable TN is approximately 1.5‰ higher. If these N forms were directly lost through flux from the soil, the resulting soil would actually become more depleted. For this reason, it is unlikely that loss of this N source would result in increased soil $\delta^{15}N$. Alternatively, it can also be assumed that P additions resulting in increased soil microbial biomass, activity, and net N mineralization (White and Reddy 2000) could significantly impact the $\delta^{15}N$ of available N forms.

Results of the bottle mineralization study demonstrate the effects of added P on N mineralization processes in the contrasting soils from sites 1 and 10 (Fig. 5). In the highly P-enriched soils of site 1, P addition at the 7.0 mg level reduced the amount of total water-extractable N (Fig. 5). The opposite effect of added P was observed in the soils of site 10, with increased amounts of total water-extractable N in both the 3.5 and 7.0 mg P treatments relative to control after the 2-week incubation (Fig. 5). The increased production of total water-extractable N in P amended soils of site 10 was not the result of increases in NH_4^+ which showed either no change in the 3.5 mg P treatment or decreased levels in the 7.0 mg P treatment relative to the non-amended control (Fig. 5). In contrast, water-extractable organic-N was higher than that of the control by 45 and 58% in the site 10 soils amended with 3.5 and 7.0 mg P, respectively (Fig. 5).

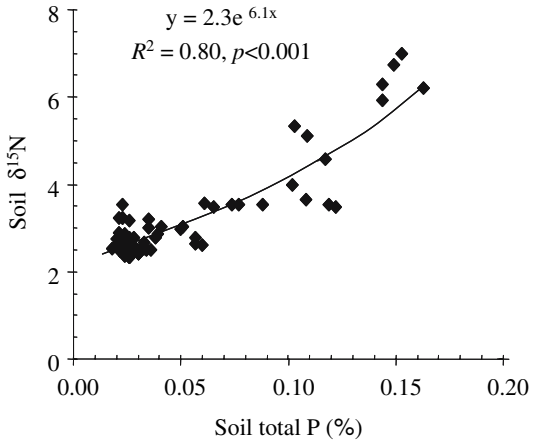

Fig. 4 Correlation of $\delta^{15}N$ with total P content in peat core samples from the impacted (1.8 km from inflows), moderately impacted (3.5 km), and non-impacted (8.1 km) WCA-2A sites

Table 1 Selected physical, nutrient, and isotopic characteristics (mean ± SD, $n = 3$) of soils used in bottle mineralization study. *wt.* Weight

Parameter	Site 1	Site 10
Moisture content (% wet wt.)	93 ± 0.3	89 ± 0.2
Ash content	12.5 ± 1.2	14.0 ± 2.1
Total C (% dry wt.)	48.3 ± 0.3	47.7 ± 2.0
Total N (% dry wt.)	3.3 ± 0.1	3.6 ± 0.2
Total P (mg kg dry wt.$^{-1}$)	1,500 ± 50	450 ± 15
Water-extractable NH_4 (μg N g soil^{-1})	81 ± 1	31 ± 0
Water-extractable organic-N (μg N g soil^{-1})	114 ± 9	95 ± 14
$\delta^{15}N$-soil-total N	5.1 ± 0.0	1.5 ± 0.0
$\delta^{15}N$-water-extractable-NH_4^+	7.7 ± 0.6	8.7 ± 1.1
$\delta^{15}N$-water-extractable-total N	4.6 ± 0.8	3.1 ± 0.3

The $\delta^{15}N$ of the net mineralized NH_4^+ ranged between 7.0 and 7.7‰ and was not significantly affected by either site or added P level (Fig. 5). There was also no effect of added P on the $\delta^{15}N$ of organic N compounds released during the 2-week bottle incubation of the site 1 soils. In contrast, there was a progressive effect of added P on the $\delta^{15}N$ of water-extractable organic N in the soils of site 10, with $\delta^{15}N$ increasing relative to the control by 1.8 and 3.0‰ in the 3.5 and 7.0 mg P treatments, respectively.

Based on the measurements of water-extractable N before and after the bottle incubation, it is clear that extractable organic N is more isotopically depleted than NH_4^+ at both WCA-2A sites. At both sites 1 and 10, extractable organic N was more isotopically depleted than the bulk soil N by ~1 to 2‰ (Fig. 5). It is also evident, that addition of P to the low-P soils of site 10 resulted in increased release of these organic N compounds. Continued loss of compounds with $\delta^{15}N$ lower than the bulk soil could result in increased $\delta^{15}N$ of the residual site 10 soil; however, the large quantity of such compounds required to enact a measurable change on the total soil N argues against this possibility. Moreover, the addition of P appeared to reverse the process in the site 10 soils by increasing the $\delta^{15}N$ of these extractable organic N to levels even above $\delta^{15}N$ of the bulk soil N.

The consistency in the $\delta^{15}N$ of NH_4^+ regardless of site or P level and effect of P to increase the $\delta^{15}N$ of organic-N released from site 10 soils, indicate that soil mineralization may not affect the $\delta^{15}N$ of soil through a loss of isotopically light N. However, the bottle study may not have been an adequate test for this mechanism. For example, the negative effect of P on N mineralization in the 7.0 mg P treatment indicates that significant N immobilization was present. In this case, any mineralized NH_4^+ would have been quickly reassimilated by the greatly increased microbial population at that high P level. Undoubtedly, the high production of NH_4^+ in the Newman et al. (2004) study occurred at much lower added P levels than even those of the 3.5 mg P treatment in the bottle incubation. For this reason, it is possible that both the 3.5 and 7.0 mg P treatments resulted in a higher N demand than that experienced during the mesocosm dosing study, and thus, the increased $\delta^{15}N$ of extractable organic N could represent the incorporation of high $\delta^{15}N$ NH_4^+.

It could also be asserted that another problem in the bottle incubation was inhibition of nitrification and denitrification. Under field conditions, the process of NH_4^+ production is not isolated, and it may also be possible that O_2 production in the water column is sufficient to allow nitrification to proceed into the surface soil. Nitrification and denitrification are both highly fractionating processes (Mariotti et al. 1981, 1988) and if present, should result in significant ^{15}N increases in the soil NH_4^+ pool. The maintenance of anaerobic conditions in this bottle technique would have prevented nitrification, and thus, did not allow sufficient enrichment in ^{15}N to occur. It is therefore possible that N mineralization, coupled with nitrification and denitrification could result in the overall enrichment of surface and/or porewater N under field conditions.

Long-term P-dosing effects on soil ^{15}N

The test of potential $\delta^{15}N$ changes as a result of P additions under field conditions lies in the isotopic analysis of samples archived from the Newman et al (2004) mesocosm dosing experiment. Because that experiment was conducted using P additions in a field setting, processes such as hydrologic transport, or coupled nitrification/denitrification would not have been excessively hindered. The experiment of Newman et al. (2004) resulted in significantly increased soil TP from ~400 to greater than 600 mg P kg^{-1} soil in the

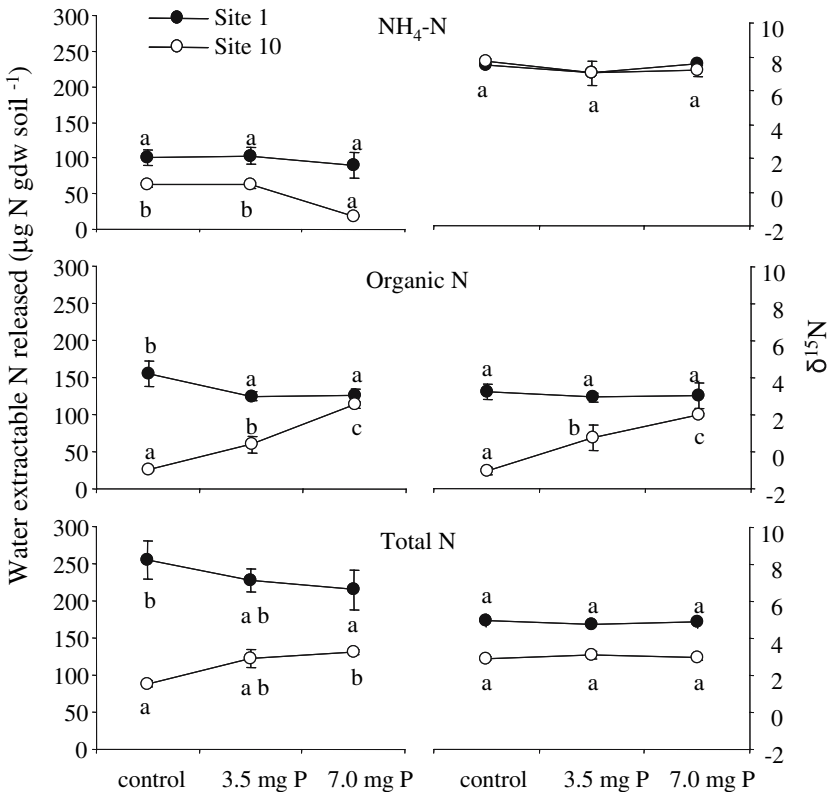

Fig. 5 Net amounts (µg N g dry weight soil^{-1}) and stable N isotopic signature (^{15}N) of N forms released from nutrient-impacted (*Site 1*) and non-impacted (*Site 10*) WCA-2A soils following 15-day bottle incubations with 0 mg (control), 3.5 mg, and 7.0 mg of added P. Points represent the means ± SD of three replicate bottles

highest dosing rate (12.8 g P m^{-2} year^{-1}) (Table 2). The increased levels of soil TP were detected after only 1 year of dosing; however, within the first few weeks after dosing, measurements of surface and porewaters indicated a significant production of NH_4^+ with levels up to 5 mg N l^{-1} higher than controls (Newman et al. 2004). There were strong gradients in concentration between the pore and floodwater suggesting a significant increase in overall N flux rates from the soil.

Despite the evidence of significant enhancement of N cycling following P loading, analysis of soil samples from the experiment of Newman et al. (2004) reveal no significant change in the overall δ^{15}N of bulk N in the 0–3 cm soil samples from the highest P-loaded mesocosms even after 5 years of loading (Fig. 6). The slight fluctuation which was measured in these soils was small (<2‰) and was consistent between dosed and control mesocosms indicating that seasonal or environmental conditions, or sampling protocols, had a greater effect on δ^{15}N in this system.

P dosing effects on macrophyte ^{15}N

Macrophyte δ^{15}N signatures may be passed down to the soil via decomposition of litter. To test this macrophyte control of soil δ^{15}N, samples of leaf and root tissues from the hydroponic growth experiment of Lorenzen et al. (2001) were analyzed for δ^{15}N. Because the experiment provided a constant supply of N at varying levels of P, the magnitude of potential reduction in plant discrimination during N uptake could be assessed. The results depicted in Fig. 7 demonstrate that as P level (and N demand) increases in these macrophytes, δ^{15}N also increases. As stated previously, increased δ^{15}N is likely the result of reduced discrimination during N uptake and assimilation by which plant N becomes more isotopically similar to the δ^{15}N of the N source (Evans 2001). By this mechanism, *Cladium* and *Typha* grown at high P levels increased the δ^{15}N of leaves and roots by as much as 12‰ relative to those grown at low P levels.

Similar shifts in δ^{15}N have been seen in macrophytes of other wetlands including mangroves (Mckee et al. 2002) and New Zealand bog species (Clarkson et al. 2005). In these cases, the increased macrophyte δ^{15}N occurred over a corresponding shift from P to N limitation similar to that in WCA-2A. McKee et al. (2002) also found that P addition increased the δ^{15}N of mangroves growing in P-limited sites and postulated that the increased δ^{15}N was not the result of a change in the δ^{15}N of the N source, but rather reflected a decreased fractionation by the mangroves as N demand increased. Clarkson et al. (2005) further enhanced this mechanistic model based on plant N demand by showing that mycorrhizal status was not responsible for observed δ^{15}N patterns of their sampled bog species.

Table 2 Values of selected nutrient parameters (total N, total C) and C:N ratios observed in soil samples (0–3 cm) from the WCA-2A long-term mesocosm P-dosing experiment (Newman et al. 2004). Values represent the mean ± SD of three replicate mesocosms except where noted

Mesocosm treatment	Year			
	1995	1996	1997	2000
	Total P (mg kg^{-1})			
12.8 g P m^{-2} $year^{-1}$	393 ± 38	624 ± 113	423 ± 129	606 ± 18
Open control	293 ± 15	398 ± 46	397 ± 21	460 ± 70
Closed control	367 ± 70	417 ± 46	401 ± 20	411 ± 40
	Total N (%)			
12.8 g P m^{-2} $year^{-1}$	3.0 ± 0.5	1.9 ± 0.2	3.0 ± 0.4	2.9 ± 0.5
Open control	3.2 ± 0.6*	2.0 ± 0.4	3.2 ± 0.6	3.3 ± 0.4
Closed control	2.8 ± 0.4	2.4 ± 0.1	3.3 ± 0.2	3.3 ± 0.5
	Total C (%)			
12.8 g P m^{-2} $year^{-1}$	34.6 ± 4.2	41.7 ± 6.2	38.2 ± 2.3	35.2 ± 3.6
Open control	36.2 ± 3.3[a]	39.5 ± 5.0	35.9 ± 4.5	38.3 ± 2.1
Closed control	36.8 ± 4.2	46.3 ± 6.2	38.4 ± 2.3	38.5 ± 3.6
	C:N (wt.:wt.)			
12.8 g P m^{-2} $year^{-1}$	11.5 ± 0.2	21.4 ± 3.0	12.9 ± 1.9	12.0 ± 1.0
Open control	11.6 ± 1.2*	19.8 ± 1.5	11.2 ± 0.6	11.6 ± 0.9
Closed control	13.6 ± 3.2	19.6 ± 2.1	11.7 ± 0.5	11.8 ± 1.0

[a] $n = 2$

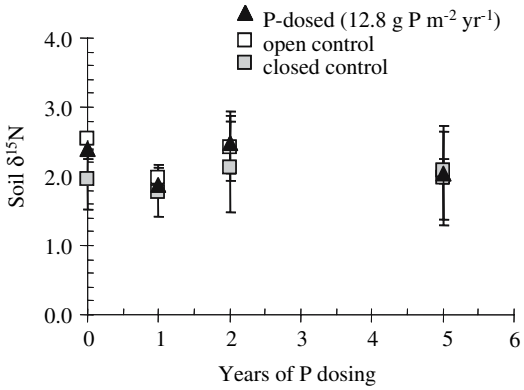

Fig. 6 Patterns of stable N isotopes ($\delta^{15}N$) of natural WCA-2A soils and those dosed with P (12 g P m^{-2} $year^{-1}$) in the mesocosm study by Newman et al. (2004). Points represent the mean ± SD of samples obtained from three replicate mesocosms

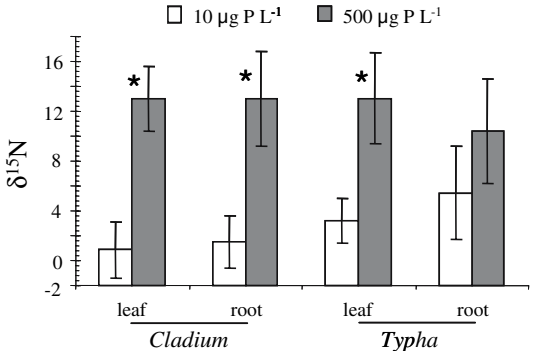

Fig. 7 Stable N isotopic ratios ($\delta^{15}N$) of native WCA-2A macrophytes (*Typha* and *Cladium*) grown in solution culture of low (10 µg l^{-1}) and high (100 µg l^{-1}) ambient P concentrations in the experiment by Lorenzen et al. (2001). *$P < 0.05$ (significantly different means; Fisher's LSD test)

The 12‰ increase in the $\delta^{15}N$ of plants grown at high P levels in this study is similar to the 13‰ increases observed by Clarkson et al. (2005), and is of a sufficient magnitude to explain the 8‰ enrichment of *Typha* in areas near the WCA-2A inflows (Inglett and Reddy 2006). Unfortunately, no samples were available from the intermediate P levels in the Lorenzen et al. (2001) hydroponic experiment, therefore, it is unclear if there are differences between $\delta^{15}N$ of *Cladium* and *Typha* at conditions of moderate N demand similar to those observed on the WCA-2A transect by Inglett and Reddy (2006). Also, we were unable to determine the isotopic composition of the N source [$(NH_4)_2SO_4$] used in the Lorenzen et al. (2001) study, therefore it is impossible to determine the exact isotopic discrimination by the two plants at high and low P levels. The consistency between *Cladium* and *Typha* in the high-P treatment may indicate the N source used in the Lorenzen et al. (2001) study was approximately 12‰. Using the measured $\delta^{15}N$ of water-extractable NH_4^+ at the impacted site 1 (Table 1), it is then reasonable to assume that at a maximum reduction in physiological discrimination, the biomass $\delta^{15}N$ of *Typha* and *Cladium* near the WCA-2A inflows would be approximately 7–8‰. This value is close to the 8‰ value recorded for $\delta^{15}N$ of live *Typha* leaves measured near the inflows (Inglett and Reddy 2006).

Conclusion

Many studies attempt to use $\delta^{15}N$ as an indicator of N cycling to identify things such as varying N sources, N pollution, or a relative increase/decrease in processes such as N_2 fixation or denitrification. In this study, live macrophytes, litter, and soils of a peat-based wetland system increased in $\delta^{15}N$ in response to P eutrophication. The observed changes in $\delta^{15}N$ were not explained by the more accepted mechanisms such as N pollution, lowered rates of N_2 fixation, or increased denitrification. For this reason, two potential mechanisms explaining P-induced changes in macrophyte and soil $\delta^{15}N$ were tested. The analysis of plants grown under constant N supply and varying P levels demonstrated that as P levels increase, the $\delta^{15}N$ of Everglades *Cladium* and *Typha* also increases (up to 12‰), likely as a result of the plants increased demand for N (Lorenzen et al. 2001). This reduction of isotopic discrimination during N uptake is consistent with general isotopic theory and may justify the biomass $\delta^{15}N$ observed for *Typha* (8‰) and *Cladium* (6‰) near the WCA-2A inflows.

In contrast to results for the macrophyte process, direct P addition to WCA-2A soils resulted in contradictory conclusions in bottle mineralization assays, and no observable change in soil $\delta^{15}N$ in a long-term field-level P dosing experiment. Based on these results, it is possible to conclude that $\delta^{15}N$ changes observed in the WCA-2A peat soils are derived from macrophyte isotopic changes which directly result from elevated P levels in the system. Despite the plausibility of the results, however, it is still important to view these results with caution as additional processes affecting macrophyte $\delta^{15}N$ (e.g., mycorrhizal status and potential fractionating effects) remain untested in the WCA-2A system.

Combined with results of other studies such as McKee et al. (2002) and Clarkson et al. (2005), these results from the Everglades seem to indicate that macrophyte physiological processes can be a dominant process in determining soil and ecosystem $\delta^{15}N$, especially in cases involving shifts in N limitation. This finding has important implications concerning N cycling in similar wetlands where P loading impacts the overall storage of N within the system. In this, $\delta^{15}N$ demonstrates that the majority of N taken up by plants is likely retained within the plant litter, and what is lost via leaching, mineralization, etc. is likely reincorporated into litter as it undergoes decomposition. The availability of P (and potentially other plant-limiting nutrients) is of key importance during this process as a determinant of the demand for N by macrophytes regulating production of biomass and microbial communities regulating N retention during peat accretion.

The results of this study also serve as another demonstration of the caution which must be exercised when interpreting observed isotopic signals. Reliance upon a single, predetermined process to explain $\delta^{15}N$ patterns should be avoided unless experimental validation can be accomplished and the effects of competing processes eliminated. In this work, the role of nutrient limitation was particularly important as P availability affected the $\delta^{15}N$ of ecosystem components. The resulting ~12‰ effect which macrophytes can exert on the overall $\delta^{15}N$ of a system is significant when compared with the typical ranges observed in global $\delta^{15}N$ patterns (Amundson et al. 2003). For this reason, other studies should also consider the potential role of non-nitrogenous nutrients (such as P and K) in affecting N demand and thus altering $\delta^{15}N$ through organic matter production.

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