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Elevated CO_2 promotes long-term nitrogen accumulation only in combination with nitrogen addition

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Abstract

Biogeochemical models that incorporate nitrogen (N) limitation indicate that N availability will control the magnitude of ecosystem carbon uptake in response to rising CO_2. Some models, however, suggest that elevated CO_2 may promote ecosystem N accumulation, a feedback that in the long term could circumvent N limitation of the CO_2 response while mitigating N pollution. We tested this prediction using a nine-year CO_2xN experiment in a tidal marsh. Although the effects of CO_2 are similar between uplands and wetlands in many respects, this experiment offers a greater likelihood of detecting CO_2 effects on N retention on a decadal timescale because tidal marshes have a relatively open N cycle and can accrue soil organic matter rapidly. To determine how elevated CO_2 affects N dynamics, we assessed the three primary fates of N in a tidal marsh: (1) retention in plants and soil, (2) denitrification to the atmosphere, and (3) tidal export. We assessed changes in N pools and tracked the fate of a ^{15}N tracer added to each plot in 2006 to quantify the fraction of added N retained in vegetation and soil, and to estimate lateral N movement. Elevated CO_2 alone did not increase plant N mass, soil N mass, or ^{15}N label retention. Unexpectedly, CO_2 and N interacted such that the combined N+CO_2 treatment increased ecosystem N accumulation despite the stimulation in N losses indicated by reduced ^{15}N label retention. These findings suggest that in N-limited ecosystems, elevated CO_2 is unlikely to increase long-term N accumulation and circumvent progressive N limitation without additional N inputs, which may relieve plant–microbe competition and allow for increased plant N uptake.

Keywords: brackish marsh, CO_2 enrichment, denitrification, isotopic biogeochemistry, nitrogen pollution, nitrogen retention and loss

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Introduction

Many ecosystems remain N-limited despite human application of 150 Tg of nitrogen (N) per year to the Earth's land surface (Schlesinger, 2009). In such ecosystems, N availability may constrain the positive growth response of vegetation to elevated CO_2 as plants remove available N from the soil N pool (Luo *et al.*, 2004). Terrestrial models that incorporate N limitation feedbacks indicate that land ecosystems may not sequester as much carbon (C) as suggested by models lacking representation of N limitation feedbacks (Hungate *et al.*, 2003; Wieder *et al.*, 2015b; Zaehle *et al.*, 2015). However, some of these models predict enhanced accrual of N through increased plant demand and reduced N losses, ultimately alleviating N limitation of the CO_2 response (Walker *et al.*, 2015). Empirical evidence exists to both support and refute this prediction; however, it is difficult to detect changes in ecosystem N mass in most terrestrial ecosystems such as forests where external flux rates are relatively small, unless assessments can be made over very long timescales (Walker *et al.*, 2015). Understanding external N fluxes is critical to accurately project long-term C storage (Wieder *et al.*, 2015a). We aimed to test the prediction that elevated CO_2 reduces N losses by measuring N accumulation (defined as N inputs—N losses) and retention (defined as the proportion remaining of a known amount of added ^{15}N) in a N-limited ecosystem with a relatively open N cycle, where external flux rates are large relative to internal flux rates and changes in N pools should be more readily detectable.

Effects of elevated CO_2 on N pools are equivocal, with some empirical evidence supporting models that predict ecosystem N accumulation (Iversen *et al.*, 2012) while other evidence indicates mixed (Reich & Hobbie, 2013) or even negative effects (Hungate *et al.*, 2014). Many CO_2 enrichment studies focus on particular pools that may not necessarily capture the trajectory of total ecosystem N (e.g. plant N in Reich & Hobbie, 2013). Few span a time period long enough to successfully detect conclusive effects on N pools, which can be small relative to considerable background variability (Walker *et al.*, 2015). Elevated CO_2 yielded an accumulation of N in belowground pools in a sweet gum plantation

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after 11 years (Iversen *et al*., 2012). In a northern mixed grass prairie, elevated CO_2 had contrasting effects on different soil N forms as measured after 3 and 4 years of CO_2 exposure, and suggested that N accrual may occur in wet years only (Carrillo *et al*., 2012). Elevated CO_2 did not affect whole-ecosystem N in a scrub oak woodland despite an increase in aboveground plant N after 11 years of exposure (Hungate *et al*., 2013). In forests, higher recoveries of ^{15}N tracers in plant tissue under elevated CO_2 likely indicated changes in internal N cycling but not necessarily changes in long-term ecosystem N retention (Zak *et al*., 2007; Hofmockel *et al*., 2011). Tidal marshes provide an excellent natural system in which to test the effects of elevated CO_2 on ecosystem N mass without the confounding influence of water availability, as they have relatively open N cycles and can accumulate organic matter faster than forest ecosystems yet respond similarly to elevated CO_2 (stimulation of NPP, periodic N limitation of CO_2 response, increase in biomass C : N ratios, Drake, 2014; Langley & Hungate, 2014; Langley & Megonigal, 2010). Wetlands are inordinately important C sinks (Mcleod *et al*., 2011), and thus, understanding how future CO_2 concentration affects N dynamics in wetlands holds importance for predicting future global C sink activity.

In addition to testing predicted changes in N accrual under elevated CO_2, we aimed to test a second hypothesis that conversion of N into relatively stable organic forms under elevated CO_2 can mitigate N pollution by reducing losses to denitrification as gaseous nitrous oxide (N_2O) and reducing N flow into surrounding water bodies. The effect of N pollution on marsh N retention is unclear and may vary depending on the status of ecosystem N limitation. Marshes that are less N-limited may become a source of N upon receiving high N inputs (Vivanco *et al*., 2015). It is also possible for marshes to maintain nearly equivalent N imports and exports, transforming N rather than changing the net ecosystem N balance (Valiela & Teal, 1979; White & Howes, 1994). The degree of N limitation is changing in many ecosystems because of the highly variable nature of anthropogenic N loading (Boyer *et al*., 2006; Ruhl & Rybicki, 2010), and interactions with other resources such as CO_2 may strengthen N demand (Luo *et al*., 2004; Langley & Megonigal, 2010) and possibly N retention (Walker *et al*., 2015). To predict how elevated CO_2 may modify the long-term fate of N pollution in the future requires understanding mechanisms of N loss such as gaseous emissions and tidal flushing.

Denitrification represents a major pathway of N loss in natural and constructed wetlands that ameliorates estuarine eutrophication (White & Howes, 1994; Hamersley & Howes, 2005; Reinhardt *et al*., 2006; Kinney & Valiela, 2013). However, denitrification can also lead to the production of N_2O, a potent greenhouse gas with a global warming potential roughly 300 times that of CO_2 on a one hundred-year horizon (Smith, 1997; Wrage *et al*., 2001). Marshes release nitrogenous gases at higher rates than many other ecosystems (Bowden, 1986), and changes in marsh denitrification rates could affect ecosystem N retention. N fertilization generally increases denitrification in most ecosystems (White & Reddy, 1999; Barnard *et al*., 2005; Koop-Jakobsen & Giblin, 2010; Niboyet *et al*., 2011). On average, elevated CO_2 also increases soil N_2O emissions (Van Groenigen *et al*., 2011); however, it can also decrease or have no effect on denitrification (Barnard *et al*., 2005; Niboyet *et al*., 2011; Brown *et al*., 2012). In N-limited terrestrial ecosystems, gross mineralization rates generally increased under elevated CO_2 likely due to increased soil C inputs by rhizodeposition and litter (Rütting & Andresen, 2015). It is possible for this same mechanism to operate in N-limited marshes, thus providing more inorganic N to nitrifying and denitrifying bacteria. Few studies have examined how elevated CO_2 and N addition interact to influence denitrification.

Tidal export is a poorly constrained mechanism of N loss in marsh ecosystems that may also be affected by global changes. Several studies report watershed or whole-ecosystem level measurements of export (Valiela *et al*., 1978; Whiting *et al*., 1987; Gribsholt *et al*., 2005); however, this route of N loss is not assessed in many manipulative studies due to challenges in measuring N mobility on a small scale. Tidal export from a *Spartina* salt marsh in New England accounted for <7% of added N for all treatments (low, high, and extra-high fertilization), which indicates that export may be a relatively minor flux of N (Brin *et al*., 2010). Factors controlling gains and losses of N in coastal ecosystems and how global change drivers, such as chronic N pollution and rising atmospheric CO_2, affect these fluxes, and overall ecosystem N storage remains unclear.

In this study, we determined how elevated CO_2 and N pollution affect the three primary fates of N in this tidal marsh: long-term retention in plants and soil, denitrification to the atmosphere, and tidal export. We tracked the fate of a ^{15}N tracer that was added in 2006 to each plot of a CO_2 by N experiment to quantify N retention in vegetation and soil, as well as to estimate lateral migration of N as an index of N mobility. Very few studies have measured plot-level N migration, and none have simultaneously examined how rising CO_2 concentration may affect the mobility, gaseous loss, and accumulation of N in marshes. To assess changes in N accumulation, we quantified N pools in plants, bulk soil, and soil porewater over time. To constrain gaseous N loss, we measured N_2O flux *in situ* and potential denitrification ($N_2 + N_2O$) with the acetylene reduction

technique using laboratory incubations of soil slurries. We predicted that: (1) elevated CO_2 would increase total ^{15}N label retention and N accumulation as suggested by some models (Walker *et al.*, 2015) primarily through enhanced plant uptake and decreased losses; (2) N addition would decrease total ^{15}N label retention and N accumulation primarily through increased mobility but also denitrification; and (3) that CO_2 and N would have additive effects when applied together.

Materials and methods

Site description and experimental design

The study took place at Kirkpatrick Marsh, a relatively unpolluted brackish marsh located along the Rhode River (38°52′26″ N, 76°32′58″W) at the Global Change Research Wetland of the Smithsonian Environmental Research Center, Edgewater, MD (Fig. 1). The C_3 sedge, *Schoenoplectus americanus*, along with two C_4 grass species, *Spartina patens* and *Distichlis spicata*, dominate plant community composition. Because of functional similarity for this study, *S. patens* and *D. spicata* are treated as a single functional group, referred to as 'grasses'. The soil is >85% organic matter to a depth of 5 m. Mean tidal range is 40 cm, and the high marsh zone is 40–60 cm above mean low water level. Salinity ranges from 4 to 15 ppt. Mean low temperature is −4 °C in January, and mean high temperature is 31 °C in July.

Twenty open-top chambers were constructed over octagonal 3.3-m^2 plots in the summer of 2005 (Langley *et al.*, 2009b). These plots were factorially exposed to two levels of atmospheric CO_2 (ambient or ambient + 340 ppm) and two levels of N addition (0 or 25 g N m^{-2} yr^{-1}) starting in May of 2006 (n = 5). Treatment abbreviations used here are as follows: control = ambient N and ambient CO_2; +N = N fertilization and ambient CO_2; +CO_2 = ambient N and elevated CO_2; N+CO_2 = N fertilization and elevated CO_2. The concentration of CO_2 added simulates moderate projections of atmospheric CO_2 for the year 2080 (Collins *et al.*, 2013), and N fertilization simulates soil N availability in more heavily polluted sites. For comparison, average N loads to the Chesapeake Bay are 14 g N m^{-2} yr^{-1} although much higher levels up to 100 g m^{-2} yr^{-1} in urban tributaries occur (Kemp *et al.*, 2005). NH_4Cl was applied to the N fertilized chambers in aliquots of 5 g m^{-2} on five occasions at approximately monthly intervals from May to September, each consisting of NH_4Cl dissolved in water taken from the Rhode River, the source of water that floods the marsh during natural tidal cycles. Fertilizer solution (5 L) was applied by backpack sprayer followed by a 5 L rinse with unamended river water. Unfertilized plots received 10 L of unamended river water, applied the same way at the same times. Pure CO_2 was mixed with a stream of ambient air and delivered through manifolds into elevated CO_2 chambers during the growing season to achieve target concentration (ambient + 340 ppm) during daylight hours (see Langley *et al.*, 2009b for further technical details).

Nitrogen-15 and total nitrogen analysis

In June of 2006, 60 mg of 99 atom% ^{15}N-NH_4Cl dissolved in 2 L of water was injected evenly from 0 to 10 cm depth to half of each plot at each intersection of a 5-cm grid to achieve a target application of 0.0634 g ^{15}N m^{-2}.

To examine N dynamics and estimate ^{15}N recovery, we measured N mass and ^{15}N label mass in above and belowground biomass throughout the study. At peak biomass in late July of each year (from 2005 to 2013), aboveground biomass was estimated using allometric equations based on stem density, height, and width for *S. americanus* and clipped subplots for *S. patens* and *D. spicata*. Three root ingrowth cores in each plot were recovered each year (2006–2013) to examine temporal patterns in ^{15}N label retention in live roots (Langley & Megonigal, 2010). Fine roots of different species were not separated. Samples of dry biomass of *S. americanus* and *S. patens* taken from clippings, and fine root biomass from all species were analyzed for [C], [N], ^{13}C, and ^{15}N composition at Smithsonian Stable Isotope Laboratory (Suitland, MD, USA) or UC Davis Stable Isotope Facility (Davis, CA, USA).

To estimate total belowground (biomass + soil organic matter) N mass and ^{15}N label mass, one soil core (6.1 cm diameter × 60 cm deep) was collected from the center of the ^{15}N-labeled zone within each chamber in 2014. The corer was 1 m long and had an open-face, semi-cylindrical chamber designed to remove an intact soil cylinder while preserving bulk density (Gouge Auger, AMS, American Falls, ID). Each core was sliced along the cutting edges of the barrel and sectioned into 2-cm depth intervals from 0 to 10 cm, 5-cm intervals from 10

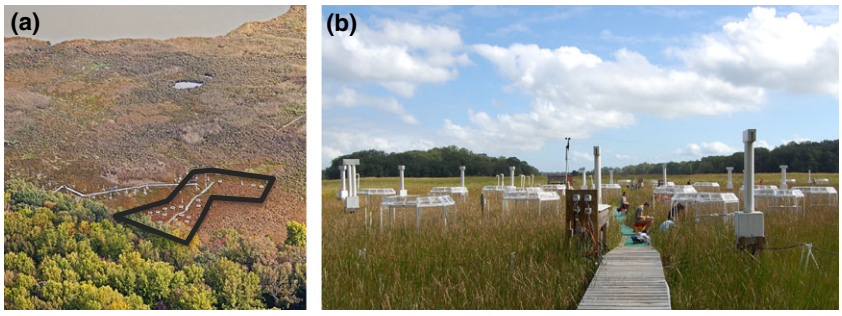

Fig. 1 View of Kirkpatrick Marsh overhead (a) and the experimental chambers used in this study (b). The outline within panel 'a' indicates the location of the experimental site used in this study.

to 30 cm, and 10-cm intervals from 30 to 60 cm. Samples were dried at 60 °C for 2 weeks. Bulk soil from each interval was ground with a mortar and pestle using liquid N_2 and analyzed for C and N concentration and isotopic composition at UC Davis Stable Isotope Facility (Davis, CA, USA). Label mass recovered for all soil and tissues was calculated as the product of mass, [N], and fraction of N derived from label according to ^{15}N composition in excess of natural abundance. Natural abundance of each tissue type was determined by prelabel ^{15}N composition and tissues from unlabeled plots.

Lateral migration

A model was developed to estimate the amount of ^{15}N label present within 1 m of each plot's labeled area (half of each plot) to a depth of 5 cm using one sample at a distance of 35 cm from the chamber. To develop this model, soil cores were collected (2.5 cm diameter × 5 cm deep) at distances of 5, 35, 50, and 100 cm outside each of three cardinal directions from the labeled zone of one chamber from each treatment. The decline in label strength ($\delta^{15}N$) with distance (d) in cm was described by an exponential decay function for each combination of chamber and direction:

$$\delta^{15}N = \alpha e^{-xd}$$

where α is the average $\delta^{15}N$ in the labeled plot. The value of x, the fitted exponential decay coefficient, was consistent among different directions and among plots of different treatments (mean ± SD = 0.0137 ± 0.0021 cm^{-1}) and was used to estimate total label migration from each plot. We extrapolated to the remaining chambers by taking one 5-cm-deep soil core at a distance of 35 cm from each chamber at the central panel only. After converting the weighted averages of $\delta^{15}N$ into label mass, we scaled to an area extending 1 m outside each labeled zone by multiplying label mass by the area of this semi-octagonal zone (8.965 m^2).

Denitrification potential

Denitrification potential was measured as described by Groffman *et al.* (1999) with slight modifications. One soil core (2.5 cm diameter × 5 cm deep) was collected from each chamber and stored on ice until being transported to Villanova University, where the cores were then refrigerated for 4 days until incubations were established. Each core was stored in a plastic bag at 4 °C with excess air removed. Water level and soil temperature for a subset of plots were recorded.

Under an O_2-free atmosphere, each sample was removed from its bag and sliced into several ~3-cm^3 cylinders, which were vigorously shaken in a 0.5-L jar with 50 mL distilled water (volume double that of the soil) to create a soil slurry. Each slurry was filtered through a screen with 1.7-mm openings in a funnel to remove roots and particles >2 mm. Ten mL of each slurry was added to separate 60 mL foil-covered, air-tight jars for a total of 20 jars. To assess potential denitrification rates, we eliminated potential C limitation and substrate limitations by adding 0.1 mL of 2.0 mM glucose, 1.0 mM KNO_3 to each jar. Jars were sealed and purged with N_2 gas at 1 Lpm for five minutes to eliminate oxygen. Acetylene gas (5 mL) was injected to inhibit N_2 production and shunt denitrification products to N_2O. Incubations were placed on a benchtop fixed-speed reciprocal shaker at 180 osc min^{-1} when not being sampled.

Samples were collected through septa at approximately 0, 1, 3, 6, and 16 h. Data collected at hour 16 were not used because the N_2O concentration plateaued before that in most time series. Headspace was sampled by plunging the syringe to mix headspace atmosphere and drawing out 3.5 mL of gas. To avoid negative pressure, we preinjected 3.5 mL N_2 gas before mixing the headspace and taking each sample. Each sample was injected into a gas chromatograph (HP 6890 GC with an ECD detector for N_2O detection) and analyzed for N_2O concentration. Rates were calculated as the slope of the linear increase in N_2O concentration over time.

In situ N_2O flux

While it is possible that N_2O flux can come from nitrifying bacteria (Wrage *et al.*, 2001), we did not distinguish between sources in this study and expect that nitrification is low given anoxic soils conditions (Herbert, 1999) and low NO_3^- at this site. In addition, in wet conditions, N_2O emissions are expected to come primarily from denitrifiers (Webster & Hopkins, 1996). Therefore, we refer to N_2O emissions as the product of denitrification herein. To estimate *in situ* denitrification rates, air-tight chambers (~1 L) were attached to two respiration collars (10-cm-diameter PVC pipe segments, implanted to a depth of 30 cm since 2006) within each plot. Overlying water partially filled each chamber, leaving ~0.5 L headspace. Four plots were used for each of 4 days balanced across the four treatments (control, +N, +CO_2, N+CO_2) for a total of 16 plots used in July. All 20 plots were used over 3 days in October and 2 days in April. Gas samples were collected in syringes (20 mL) from each chamber after pre-injection of air (20 mL) and mixing of headspace (4–6 samples per time series) over the course of ~2 h. Headspace was mixed before samples were taken. Gas samples were immediately transported back to the laboratory and transferred into 12-mL vials with screwcaps and septa (Labco Ltd., Exetainer Brand, Lampeter, Ceredigion, UK) that were previously flushed with N_2 (as a precaution) and then evacuated with a vacuum pump (to increase the likelihood of N_2O detection). Samples were analyzed for N_2O concentration on a gas chromatograph and autosampler (Varian 450 GC with a CTC Analytics CombiPAL autosampler and an ECD detector for N_2O detection, FID detector for CH_4 detection, and TCD detector for CO_2 detection). Individual chamber height above ground, corresponding water level, and the number of stems within respiration collars were measured for each plot at the time of gas sampling. Water, soil, and air temperature were also recorded.

Porewater NH_4^+ concentration

We measured porewater [NH_4^+] as an index of N availability. Although porewater contains a small mass of N compared to other ecosystem pools, mineral porewater [N] integrates the effects of major ecosystem fluxes, and accurately reflects

ecosystem N availability to plants and microbes. Porewater was sampled at least three times per growing season through nine total wells, three representing each depth of 20, 40, and 80 cm in each experimental plot (Langley *et al.*, 2009a). Porewater samples were pooled within each depth and were analyzed for ammonium concentration by the Bertholet Reaction according to EPA Method 350.2 (USEPA, 1979). In these anoxic soils, porewater nitrate (NO_3^-) is typically below detection limits and does not contribute substantially to total mineral [N].

Statistical analyses

Normality was assessed using the Shapiro–Wilk test, and homogeneity of variances was assessed using Bartlett's test. Data were transformed using the natural log as needed. Treatment effects were tested using two-way ANOVAs. Two-way repeated measures MANOVAs were used for aboveground, fine root, and total plant ^{15}N label mass and N mass, as well as porewater [NH_4^+]. When significant interactions were found, Tukey's HSD *post hoc* test was used for pairwise comparisons. Statistical analyses were performed using RSTUDIO version 0.98.490 (R Core Team, 2013) and JMP PRO 11.0.0 (SAS Institute Inc., Cary, NC, USA).

Results

^{15}N label retention

Elevated CO_2 had no significant effect on total ^{15}N label retention (Table 1). There was no significant effect of elevated CO_2 on ^{15}N label mass aboveground in 2013 (Fig. 2). Most recovered ^{15}N label was found within the top 10 cm of soil across all treatments (Fig. 3b). Elevated CO_2 had no significant effect on ^{15}N label mass at any soil depth (Table S2) or in fine roots (Table S1).

N addition significantly decreased total retention of the added ^{15}N label by 55.5% compared to the control (two-way ANOVA, $F_{1,15} = 31.204$, $P < 0.0001$) primarily through a 56.6% reduction belowground (Fig. 2). N addition significantly reduced ^{15}N label near the soil surface at all intervals from 0 to 10 cm (Fig. 3b, Table S2). Interestingly, +N plots consistently contained more ^{15}N label below 2 cm than N+CO_2 plots, yet both contained about the same amount belowground total (13.7 ± 3.3 mg ^{15}N label m^{-2} for +N plots and 14.9 ± 2.9 mg ^{15}N label m^{-2} for N+CO_2 plots, Figs 2, 3). A significant NxTime interaction indicates that N addition also significantly reduced ^{15}N label mass in fine roots compared to the control, but that the magnitude of this difference decreased through time (Fig. 4g, Table S1, two-way repeated measures MANOVA, NxTime: $F_{7,10} = 7.223$, $P < 0.001$). Effects on total ^{15}N label retention in the N+CO_2 treatment were driven primarily by N addition, as belowground retention decreased significantly by 51.2% compared to the control (Fig. 2).

Table 1 Two-way ANOVAs of ^{15}N label retention and recovery

Test	Factor	*F*	df_{num}	df_{denom}	*P*
Belowground	CO_2	0.032	1	15	0.860
	N	23.490	1	15	**<0.001**
	CO_2*N	0.095	1	15	0.763
Aboveground	CO_2	2.322	1	16	0.147
	N	1.148	1	16	0.300
	CO_2*N	0.002	1	16	0.962
Migration	CO_2	2.119	1	16	0.165
	N	0.280	1	16	0.604
	CO_2*N	0.170	1	16	0.686
Total Retention	CO_2	0.064	1	15	0.804
	N	31.204	1	15	**<0.001**
	CO_2*N	0.487	1	15	0.496

Bold *P*-values are statistically significant at $P < 0.05$.

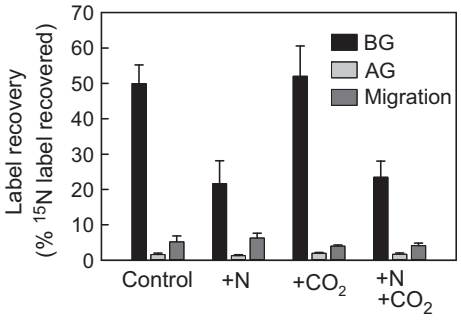

Fig. 2 Average percent ^{15}N label recovery across treatments belowground (BG) (soil with roots included), aboveground (AG), and within 1 m of labeled zone to a depth of 5 cm (Migration). BG and AG were scaled to the area of the labeled zone, which is half of the plot. Migration was scaled to the area 1 m outside of the labeled zone. No significant differences were found in ^{15}N label migration or aboveground retention by two-way ANOVA. N addition significantly reduced belowground ^{15}N label retention (two-way ANOVA, $F_{1,15} = 23.490$, $P < 0.001$). Error bars represent standard error.

Overall, *S. americanus* retained more ^{15}N label than grasses. *S. americanus* retained an average of 7107–1054 ug ^{15}N m^{-2} from 2006 to 2013 (Fig. 4e), while the grasses lost nearly all ^{15}N label, declining from an average of 2048 to 28 ug ^{15}N m^{-2} (Fig. 4f). Total ^{15}N label recovery, which includes within-plot ^{15}N label and ^{15}N label that migrated laterally belowground to a distance one meter from the labeled zone, ranged from 29.2 to 58.0% of ^{15}N initially added. N addition significantly decreased total ^{15}N label recovery (two-way ANOVA, $F_{1,15} = 25.631$, $P < 0.001$).

Migration

N addition increased ^{15}N label migration by 21% while elevated CO_2 decreased migration by 23% on average, although the differences were not significant (Fig. 2,

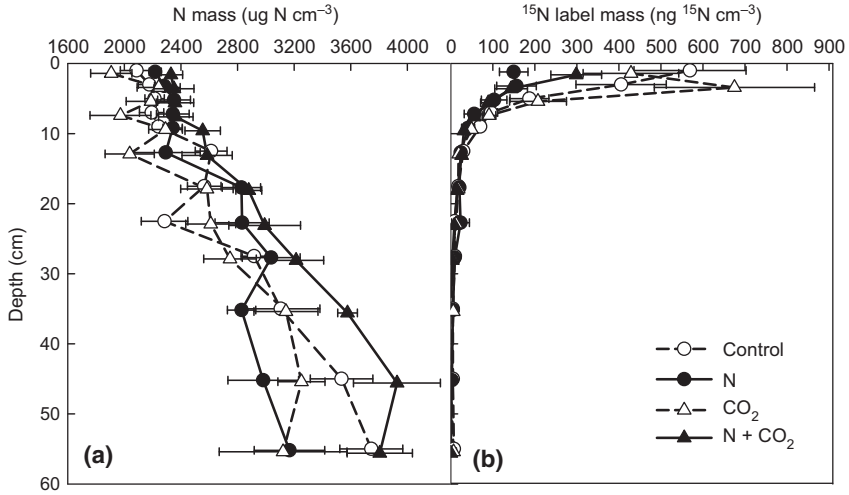

Fig. 3 Average N mass (a) and ^{15}N label mass (b) to a depth of 60 cm (N = 19) belowground (bulk soil including roots). N addition decreased ^{15}N label mass in the top 10 cm. Results of two-way ANOVAS for each depth interval are shown in Tables S2 and S4. Error bars represent standard error. Note that points are slightly offset to improve clarity.

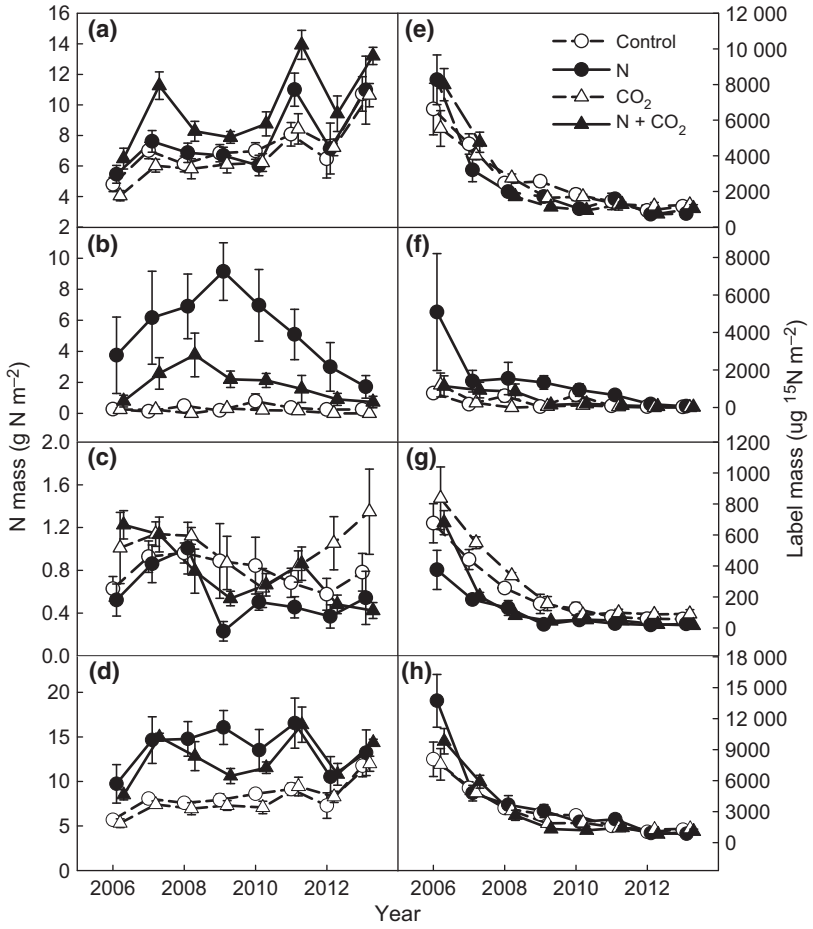

Fig. 4 Above and belowground plant N mass (a–d) and ^{15}N label mass (e–h) over eight years. N mass (g N m^{-2}) in *S. americanus* shoots (a), *S. patens* and *D. spicata* shoots (b), fine roots (c), and total plant (d). ^{15}N label mass (ug ^{15}N m^{-2}) in *S. americanus* stems (e), *S. patens* and *D. spicata* stems (f), fine roots (g), and total plant (h). Total plant measurements do not include coarse roots. Note that Y-axes vary to improve clarity. Results of two-way repeated measures MANOVAS are reported in Tables S1 and S3.

Table 1). Interestingly, migration under the N+CO_2 treatment more closely mirrored the trend under elevated CO_2 than N addition alone, leading to a 21% reduction in migration.

Aboveground and belowground N pools

Elevated CO_2 alone did not have a significant effect on total plant N mass but did affect aboveground plant N mass in combination with N addition, strengthening the increase in aboveground N mass under N addition in *S. americanus* (Fig. 4a) while dampening the increase under N addition in grasses (Fig. 4b) as described below. N addition significantly increased total plant N mass in most years (Fig. 4d,h). The effect of N addition alone on aboveground N mass was stronger in grasses; however, CO_2 and N interacted for *S. americanus* (Table S3) as N addition only significantly increased N mass under elevated CO_2 for this species (Fig. 4a,b, two-way repeated measures MANOVA, CO_2xN: $F_{1,16} = 0.360$, $P = 0.029$). In contrast, the CO_2xN interaction for grasses (Table S3) indicated that elevated CO_2 dampened the effect of N addition on aboveground N mass (two-way repeated measures MANOVA, CO_2xN: $F_{1,16} = 0.555$, $P = 0.001$).

Elevated CO_2 significantly affected N mass of fine roots, generally causing an increase (Fig. 4c, two-way repeated measures MANOVA, $F_{1,16} = 0.438$, $P = 0.018$) while N addition generally decreased N mass of fine roots (Fig. 4c, two-way repeated measures MANOVA, $F_{1,16} = 0.530$, $P = 0.010$). Belowground N mass was significantly increased by elevated CO_2 at a depth of 30–40 cm (Table S4). N addition increased belowground N mass only at depths of 15–25 cm (Table S4). Together, CO_2 and N interacted to increase total belowground N mass (to a depth of 60 cm) in soil compared to either treatment alone (two-way ANOVA, CO_2xN: $F_{1,15} = 9.391$, $P = 0.008$). From 40 to 50 cm, the combination of elevated CO_2 and N addition increased belowground N mass, but either treatment alone decreased belowground N mass (two-way ANOVA, CO_2xN: $F_{1,15} = 6.218$, $P = 0.025$).

Table 2 Two-way ANOVAS of denitrification rates

Test	Factor	*F*	df_{num}	df_{denom}	*P*
Denitrification potential	CO_2	0.517	1	16	0.482
	N	1.699	1	16	0.211
	CO_2*N	0.210	1	16	0.653
In situ N_2O Flux July (2014)	CO_2	2.027	1	12	0.180
	N	9.061	1	12	**0.011**
	CO_2*N	7.329	1	12	**0.019**
In situ N_2O Flux Oct. (2014)	CO_2	0.036	1	16	0.852
	N	2.184	1	16	0.159
	CO_2*N	0.743	1	16	0.401
In situ N_2O Flux April (2015)	CO_2	0.019	1	16	0.891
	N	0.735	1	16	0.404
	CO_2*N	1.353	1	16	0.262

Bold *P*-values are statistically significant at $P < 0.05$.

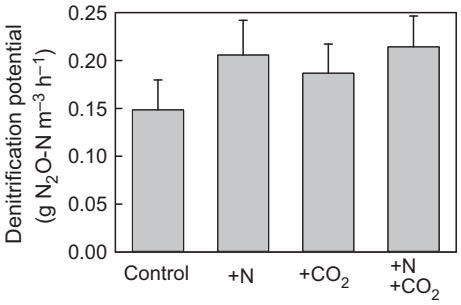

Fig. 5 Denitrification potential across treatments relevant to a depth of 5 cm (N = 20). No significant differences were found by two-way ANOVA. Error bars represent standard error.

Denitrification

There were no significant treatment effects on denitrification potential (Table 2), which averaged 0.189 ± 0.032 g N_2O-N m^{-3} h^{-1} across treatments (Fig. 5). However, there were tendencies of higher denitrification potential in communities from +CO_2 plots and +N plots (Fig. 5).

In situ N_2O emissions were higher in July than October yet similar between July and April across all treatments (Fig. 6). Elevated CO_2 and N addition interacted to affect N_2O flux in July (two-way ANOVA, $F_{1,12} = 7.329$, $P = 0.019$). N addition at ambient CO_2 increased N_2O flux in July to 8.55 ± 1.29 ug N_2O-N m^{-2} h^{-1} on aver-

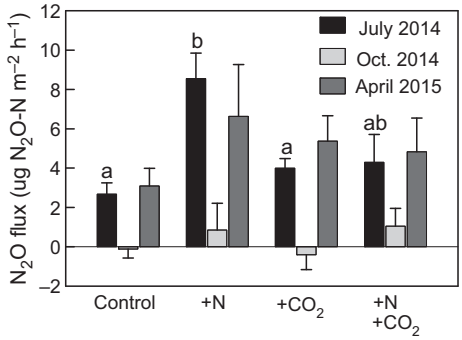

Fig. 6 N_2O flux *in situ* across treatments in three seasons (July: N = 16, Oct. and April: N = 20). For July, a significant interaction of CO_2xN was found by two-way ANOVA ($F_{1,12} = 7.329$, $P = 0.019$) and pairwise comparisons are indicated above. Columns that do not share a letter are significantly different from one another for July data. For October and April, no significant differences were found by two-way ANOVA. Error bars represent standard error.

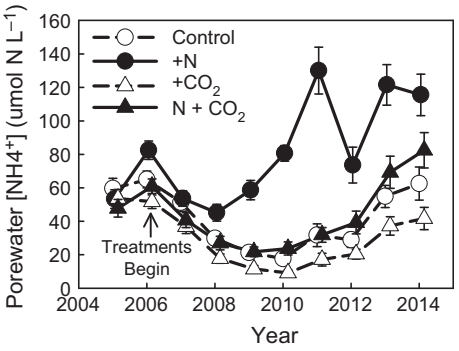

Fig. 7 Porewater NH_4^+ concentration through time. Each mean represents $[NH_4^+]$ averaged across months and depths. Means at each time point are offset to minimize overlapping error bars. Treatment application began in 2006. Results of two-way repeated measures MANOVAS are reported in table S5. Error bars represent standard error.

age, a rate over triple that measured in control plots, while elevated CO_2 alone had a nonsignificant tendency to increase denitrification by 49% to a rate of 3.99 ± 0.49 ug N_2O-N m^{-2} h^{-1} on average. The effect of N addition was reduced by elevated CO_2 in July and April, so that rates only increased by an average of 61.1% in July and 56.5% in April compared to the control, although the increases were not significant. October N_2O flux did not differ significantly across treatments but was generally higher with N addition at both CO_2 concentrations (Fig. 6). April N_2O flux did not differ significantly across treatments but showed the same trends in treatment averages as July N_2O flux (Fig. 6).

Porewater NH_4^+ concentration

Porewater $[NH_4^+]$ averaged by time and depth was highest in +N plots, where it decreased for the first 3 years of NH_4Cl application but then rapidly increased (Fig. 7). N addition significantly increased porewater $[NH_4^+]$ at depths of 20 and 40 cm with a marginally significant effect at 80 cm (two-way repeated measures MANOVAS, 20 cm: $F_{1,16} = 16.134$, $P = 0.001$; 40 cm: $F_{1,16} = 10.859$, $P = 0.005$; 80 cm: $F_{1,15} = 3.679$, $P = 0.074$). Porewater $[NH_4^+]$ in all other treatments, including control plots, decreased for the first 4–5 years after treatment application began and then started to slowly increase (Fig. 7). Elevated CO_2 significantly decreased porewater $[NH_4^+]$ regardless of N treatment (Fig. 7, two-way repeated measures MANOVA, $F_{1,16} = 7.758$, $P = 0.014$), and this effect was significant at each depth (Table S5). This reduction was particularly dramatic where N was also added (compare +N to N+CO_2 in Fig. 7, Table S5). In the N+CO_2 treatment, porewater $[NH_4^+]$ started out slightly below the control but began to exceed the control by 2013 (Fig. 7).

Discussion

Elevated CO_2 alone reduced N availability compared to the control to a depth of 80 cm, but did not increase plant N uptake, N accumulation, or ^{15}N label retention. N addition strongly decreased ^{15}N label retention regardless of CO_2 treatment. Interestingly, CO_2 and N addition interacted to increase N mass more than addition of either resource alone. We discuss how these results address our three predictions and explore the underlying mechanisms in detail below.

N retention and accumulation under elevated CO_2

We hypothesized that elevated CO_2 would increase ^{15}N label retention and N accumulation by increasing plant N uptake and reducing N losses. That elevated CO_2 reduces N availability underlies the progressive N limitation hypothesis (Luo *et al*., 2004). This notion of 'tighter' N cycling under elevated CO_2, along with ecosystem models that are structured to capture it, has engendered predictions that elevated CO_2 will reduce N losses and increase long-term N accumulation, ultimately offsetting progressive N limitation (Walker *et al*., 2015). In the present study, elevated CO_2 consistently reduced porewater N concentrations to a depth of 80 cm (Fig. 7), indicating lower N availability as in other CO_2 studies in herbaceous ecosystems (Hovenden *et al*., 2008; Carrillo *et al*., 2012). However, the consistent decrease in porewater $[NH_4^+]$ under elevated CO_2 at ambient N (Fig. 7) was not accompanied by increased N mass in plant or soil pools (Table 3, Figs 3, 4) or by increased retention of the ^{15}N label. The lack of a belowground response in ^{15}N label retention to elevated CO_2 alone corroborates findings in a semi-arid grassland (Dijkstra *et al*., 2008). These results counter the hypothesis that rising atmospheric CO_2 concentration will stimulate N accumulation and retention by increasing plant N demand and reducing N losses, but perhaps only in ecosystems where plants are highly N-limited and therefore may not strongly respond to elevated CO_2, as discussed below.

In contrast, N accumulation was significantly higher in N+CO_2 plots compared to under either treatment alone (Table 3), suggesting that elevated CO_2 may only cause N accumulation when additional N inputs are provided in strongly N-limited ecosystems, as plant–microbe competition may constrain plant responses to elevated CO_2. Indeed, total plant N uptake increased in N+CO_2 plots relative to the control (Fig. 4d), supporting the prediction that increased plant N uptake under

Table 3 N mass ± standard error (g m^{-2}) for key pools in year 8 sorted by treatment

	Control	+N	+CO_2	N+CO_2
Aboveground plant	10.97 ± 1.25	12.68 ± 2.95	10.64 ± 0.76	13.93 ± 0.94
Belowground bulk soil including roots (to 60 cm)	1775.6 ± 72.3	1668.0 ± 70.9	1662.3 ± 75.3	1957.3 ± 39.3
Soil porewater (to 1 m)	0.77 ± 0.09	1.70 ± 0.17	0.52 ± 0.08	0.97 ± 0.13
Estimated cumulative denitrification (N_2+N_2O) *	0.20 ± 0.09 to 2.04 ± 0.88	0.65 ± 0.20 to 6.52 ± 1.97	0.30 ± 0.07 to 3.04 ± 0.75	0.33 ± 0.22 to 3.28 ± 2.15

*The range of cumulative denitrification (N_2O+N_2) was estimated by assuming both a N_2O : N_2 ratio of 1 : 19 and a more conservative ratio of 1 : 1. The 1 : 19 ratio is an approximation of the ratio expected for heavily N polluted estuarine sediments (Seitzinger & Kroeze, 1998) although ratios can vary between 1 : 19 and 1 : 1 in salt marsh sediments (Lee *et al.*, 1997). We extrapolated rates measured in July across four-month growing seasons over 8 years.

elevated CO_2 may increase N accumulation. We caution that this response is not general to every ecosystem and is unlikely to apply to naturally N-rich ecosystems. Furthermore, while the marsh offers a chance to explore N dynamics under elevated CO_2 without the confounding influence of water availability, differences in soil moisture and O_2 are likely to affect responses to elevated CO_2 in other ecosystems. For example, elevated CO_2 could increase soil moisture and reduce N constraints in a semi-arid ecosystem without additional N inputs, provided N availability is low due to low N mineralization (as in Dijkstra *et al.*, 2008). Importantly, we also found that increased plant uptake and N accumulation were not accompanied by reduced N losses, as indicated by the ^{15}N label (Fig. 2).

Our findings indicate that elevated CO_2 could at once strengthen plant N uptake while stimulating N losses when ecosystems receive inputs of additional N. The primary route of N loss in N+CO_2 plots is unclear based on our results, as we did not see a significant stimulation in either ^{15}N label migration or N_2O flux. However, our migration measurements did not capture all ^{15}N label that has migrated and our N_2O flux measurements only represent snapshots in time. It is plausible that higher rates do occur, as wetlands can be characterized by low denitrifying activity while still containing microsites of high activity based on microscale gradients in soil resource availabilities (Orr *et al.*, 2014). It is also possible that N_2 emission, which we did not measure *in situ*, was stimulated disproportionately to N_2O emission or that we missed the 'hot moment' when pulses of NO_3^- occurred. Elevated CO_2 consistently boosts fine root productivity at this site (Langley *et al.*, 2009a) and yields higher concentrations of dissolved organic carbon in soil porewater (Keller *et al.*, 2009), likely indicating greater delivery of labile C to the extensive rhizosphere, which could promote denitrification Baggs *et al.*, 2003). Moreover, elevated CO_2 can stimulate rhizosphere oxidation (Wolf *et al.*, 2007). Enhanced microbial N acquisition due to rhizodeposition of C, along with enhanced oxygenation, could support greater nitrification and ultimately N loss to denitrification.

The significant reduction in porewater [NH_4^+] under elevated CO_2 alone was unexpected given no change in ^{15}N label retention or N accumulation. The error in total soil N was great compared to the treatment effect size of CO_2 on porewater N (Table 3), so there could be accumulation that was not detected. However, plant N pools, a more sensitive measure, did not suggest increased plant uptake either (Table 3). In fact, elevated CO_2 alone tended to decrease both soil and plant N pools. While rates of N_2O flux were high enough to cause the consistent depression of porewater [NH_4^+]

observed, elevated CO_2 did not significantly stimulate *in situ* N_2O flux (Figs 5, 6). In addition, the potential of the denitrifying communities across treatment groups is similar, although it is possible for elevated CO_2 to change microbial communities and their function (Osanai *et al*., 2015). Furthermore, we did not observe a decrease in ^{15}N label retention under elevated CO_2 alone, which would be expected if N losses increased (as in Hungate *et al*., 2013). It is perhaps more likely that lower canopy-level transpiration could have reduced bulk flow of deeper (below 80 cm) porewater [NH_4^+] to the surface (Mcdonald *et al*., 2002). Decreased canopy-level transpiration under elevated CO_2 has been documented in a nearby CO_2 enrichment study in this wetland (Li *et al*., 2010) and live roots extend beyond 80 cm in these plots (personal observation). Although total denitrification rates remain a highly uncertain component of the N cycle at this site, our results suggest that rising CO_2 will not change, let alone increase, N accumulation or retention by strongly N-limited marshes without additional N inputs.

Responses to N addition at ambient CO_2

Marshes currently receiving high N inputs may retain a lower proportion of N according to our findings, which corroborates other studies in wetland ecosystems (Templer *et al*., 2012). As predicted, N addition decreased total plot ^{15}N label retention and was driven by reduced belowground retention (Fig. 2, $F_{1,15} = 23.490$, $P < 0.001$). In addition, belowground ^{15}N label loss under N addition was only significant in the top 10 cm of soil (Table S2), despite no significant change in N mass (Fig. 3, Table S4). This pattern indicates that N addition accelerated N turnover in +N plots from 0 to 10 cm, with high inputs stimulating high losses. Belowground retention was more important than aboveground retention after 9 years of N addition despite the large reduction in absolute belowground ^{15}N retention. This pattern corroborates results from a New England marsh in which 40% of added ^{15}N was ultimately buried in belowground organic matter after 7 years (White & Howes, 1994). Moreover, belowground retention dominated across all treatments (accounting for 21.7–52.0% of ^{15}N initially added), indicating the importance of belowground storage in the long term.

Although N addition did not significantly affect total aboveground (all species) ^{15}N label retention after 8 years of N fertilization (Fig. 2), ^{15}N label mass was significantly higher in +N plots relative to the control in grasses in most years (Fig. 4f, Table S1). However, *S. americanus* dominated the plots and had a superior ability to sequester the ^{15}N label overall, thereby overriding enhanced ^{15}N in grasses relative to the control under N addition (Fig. 4e,f). Greater retention by *S. americanus* is likely due to internal seasonal recycling between stems, roots, and rhizomes. Yet, when grasses flourished under N addition, N uptake was suppressed in *S. americanus* (Fig. 4a,b). However, the decline in grass biomass after 2009 was due to a rise in sea level (as observed in Langley *et al*., 2013), rendering N more available to the more flood-tolerant sedge. Overall, changes in belowground ^{15}N label retention due to N addition affected total ^{15}N label retention much more than the average changes in aboveground ^{15}N label mass or ^{15}N label migration. The fate of the unrecovered ^{15}N label is unclear, but it must have migrated beyond our out-of-plot measurements or been emitted as gas, given that losses to volatilization and herbivory are likely to be negligible in this marsh.

Nitrogen loss to export, defined herein as lateral or vertical movement of N by diffusion or bulk flow, likely accounted for a greater portion of the decrease in belowground N retention in +N plots than observed. While it appears that very little ^{15}N label migrated, our measurements could not capture all ^{15}N label that migrated outside of the plots. Tidal flushing likely removed N well beyond the range of our measurements. In addition, some vertical loss over time was missed as our out-of-plot measurements only account for the top 5 cm of soil. Although our inability to capture all exported ^{15}N label could have dampened treatment differences, our estimates of migration likely do capture the relative mobility of the ^{15}N label across different treatments. In contrast, it is possible that relative trends across treatments become weaker at depths below our measurements. However, the high input of excess N appeared to saturate the capacity of the system to accumulate N via biotic uptake and soil sorption sites after 4 years of N application under ambient CO_2 (Fig. 7). We hypothesize that some of the ^{15}N label was displaced by NH_4^+ sorbing onto cation exchange sites, causing reduced ^{15}N label retention within plots and the tendency of greater ^{15}N label migration outside +N chambers (Fig. 2). We suspect that the capacity of the marsh to mitigate N pollution through N retention declines with N loading and that a large portion of N may be lost with outflowing tidal waters, where it would contribute to algal blooms that release toxins, the loss of submerged aquatic vegetation, and the formation of anoxic dead zones (Bowen & Valiela, 2001; Kemp *et al*., 2005; Bricker *et al*., 2008).

N addition stimulated N_2O flux as expected and was likely a route of ^{15}N loss as well (Fig. 6). Added NH_4^+ may have alleviated plant–microbe competition, stimulating nitrification and thus the production of NO_3^- for denitrifying bacteria. Our observation of higher N_2O emission with N addition is consistent with other stud-

ies showing that N stimulates denitrification in marsh ecosystems (Hamersley & Howes, 2005; Koop-Jakobsen & Giblin, 2010). Extrapolating *in situ* rates since the time of ^{15}N label application suggests that N_2O emissions could theoretically account for a large portion of the ^{15}N label loss (Table 3).

Will elevated CO_2 help mitigate N pollution?

We found a positive interaction of CO_2 and N on ecosystem N accumulation leading us to reject our hypothesis that the two effects would be additive. This finding indicates that although elevated CO_2 alone did not increase N accumulation, it may encourage N accumulation where N inputs are high such as polluted marshes. Indeed, the ecosystem N mass difference between N+CO_2 and control is roughly equivalent to the cumulative amount added over the course of the study, indicating that elevated CO_2 allowed the marsh to sequester a large portion of added N even with N losses much greater than in unfertilized plots, as indicated by ^{15}N label loss. Increased N inputs outweighed the stimulation of N loss, resulting in N accumulation.

Despite a large difference in N mass between +N and N+CO_2 plots, there was no difference in ^{15}N label retention, which indicates that elevated CO_2 did not reduce N loss rates. This apparent discrepancy could be explained by spatial and temporal differences in the ^{15}N label retention and N accumulation. A large portion of ^{15}N label mass was accounted for in shallow soil (<10 cm deep, Fig. 3b) regardless of treatment, while the greatest treatment effects on belowground N mass occurred at depths from 15 to 50 cm (excluding 25–30 cm, Table S4, Fig. 3a). The ^{15}N label was added at a single time point before treatments exhibited strong effects while the N addition treatments have been applied each year as treatment effects have accumulated. For instance, according to differences in porewater [NH_4^+], N addition appeared to saturate biotic demand and soil exchange sites in the +N plots beginning around 2009 while [NH_4^+] in N+CO_2 plots has not statistically surpassed that of control plots (Fig. 7). Differences in ecosystem N status over time could lead to different fates of the ^{15}N label and added N.

Taken together, our findings show that elevated CO_2 alone did not affect N retention, accumulation, or losses in a relatively unpolluted marsh for 9 years. Elevated CO_2 elicited plant N uptake and ecosystem N accumulation only where N was added, suggesting that while rising CO_2 may increase plant N demand, which should lead to N accumulation in ecosystems, plant access to N may be decreased in N-limited ecosystems (similar to findings of Feng *et al.*, 2015) particularly in the absence of water limitation and if elevated CO_2 intensifies plant–microbe competition. Therefore, initial N constraints on responses to elevated CO_2 may prevent the increased plant N uptake and concomitant N accumulation that may alleviate PNL in the long term. Furthermore, enhanced N accumulation under the combination of N addition and elevated CO_2 did not reduce N losses and may have stimulated N mobility or microbially mediated N losses through processes such as nitrification and denitrification. Based on our results, N polluted marshes will retain a smaller fraction of N inputs due to stimulation in N_2O flux and/or N export as biotic N demand and soil sorption sites become saturated, contributing to both global warming and eutrophication of coastal waters. Yet, as CO_2 concentration rises, these additional N inputs may enhance ecosystem N accumulation despite higher N losses and sustain plant responses to elevated CO_2.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Two-way repeated measures MANOVAs of aboveground and fine root ^{15}N label mass from 2006 to 2013.
Table S2. Two-way ANOVAs of ^{15}N label mass by depth intervals.
Table S3. Two-way repeated measures MANOVAs of N mass from 2006 to 2013.
Table S4. Two-way ANOVAs of N mass by depth intervals.
Table S5. Two-way repeated measures MANOVAs of porewater [NH_4^+] from 2006 to 2014.