UC Berkeley
UC Berkeley Previously Published Works

Title

Use of stable nitrogen isotopes to track plant uptake of nitrogen in a nature-based treatment system

Permalink

https://escholarship.org/uc/item/1pp6d7s0

Authors

Cecchetti, Aidan R
Sytsma, Anneliese
Stiegler, Angela N
et al.

Publication Date

2020-12-01

DOI

10.1016/j.wroa.2020.100070

Peer reviewed

Use of stable nitrogen isotopes to track plant uptake of nitrogen in a nature-based treatment system

Aidan R. Cecchetti [a, c], Anneliese Sytsma [b, c], Angela N. Stiegler [a, c], Todd E. Dawson [d, e], David L. Sedlak [a, c, *]

[a] Department of Civil & Environmental Engineering, University of California Berkeley Berkeley, CA 94720, United States
[b] Department of Landscape Architecture and Environmental Planning, University of California Berkeley Berkeley, CA, 94720, United States
[c] US National Science Foundation Engineering Research Center (ERC) for Re-Inventing the Nation's Urban Water Infrastructure (ReNUWIt), United States
[d] Department of Integrative Biology, University of California Berkeley Berkeley, CA, 94720, United States
[e] Center for Stable Isotope Biogeochemistry, University of California Berkeley Berkeley, CA, 94720, United States

A B S T R A C T

In nature-based treatment systems, such as constructed wetlands, plant uptake of nutrients can be a significant removal pathway. Current methods for quantifying plant uptake of nitrogen in constructed wetlands, which often involve harvesting biomass and assuming that all nitrogen stored in plants was derived from wastewater, are inappropriate in pilot- and full-scale systems where other sources of nitrogen are available. To improve our understanding of nitrogen cycling in constructed wetlands, we developed a new method to quantify plant uptake of nitrogen by using stable isotopes and a mixing model to distinguish between nitrogen sources. We applied this new method to a pilot-scale horizontal levee system (i.e., a subsurface constructed wetland) over a two-year monitoring period, during which 14% of nitrogen in plants was wastewater-derived on average and the remaining plant nitrogen was obtained from the soil. Analysis of nitrogen isotopes indicated substantial spatial variability in the wetland: 82% of nitrogen in plants within the first 2 m of the slope came from wastewater while less than 12% of plant nitrogen in the remainder of the wetland originated from wastewater. By combining these source contributions with remote-sensing derived total biomass measurements, we calculated that 150 kg N (95% CI = 50 kg N, 330 kg N) was taken up and retained by plants during the two-year monitoring period, which corresponded to approximately 8% of nitrogen removed in the wetland. Nitrogen uptake followed seasonal trends, increased as plants matured, and varied based on design parameters (e.g., plant types), suggesting that design decisions can impact this removal pathway. This new method can help inform efforts to understand nitrogen cycling and optimize the design of nature-based nutrient control systems.

1. Introduction

Humans have extensively modified the nitrogen cycle (Galloway et al., 2008), which has caused widespread damage to aquatic ecosystems, such as eutrophication (Sutton and Bleeker, 2013; Freeman et al., 2019). These impacts have been exacerbated by the loss of coastal wetlands (Li et al., 2018) that historically have reduced the export of nutrients from terrestrial systems (Megonigal and Neubauer, 2019). Despite past attempts to control anthropogenic nutrient inputs using conventional management methods, eutrophication has frequently persisted (Thornton et al., 2013). As an alternative to conventional approaches, engineers have begun to consider the use of constructed wetlands to manage nutrient discharges. Horizontal levees are a new type of subsurface constructed wetland that can protect coastal urban areas against flooding, while treating wastewater effluent in managed wetland habitats (Cecchetti et al., 2020a). These new types of constructed wetlands can also provide restored habitat and a suite of additional benefits (Kadlec and Wallace, 2009; Cecchetti et al., 2020a). Their low cost and resource requirements relative to conventional single-benefit solutions make constructed wetlands particularly attractive to utility managers (Kadlec and Wallace, 2009; Harris-Lovett et al.,

* Corresponding author. Department of Civil & Environmental Engineering, University of California Berkeley Berkeley, CA 94720, United States.
  E-mail address: sedlak@berkeley.edu (D.L. Sedlak).

2019).

Despite these benefits, there remain many uncertainties about the performance of horizontal levees and the exact processes by which they remove nutrients. In subsurface wetlands, nitrogen is often removed or immobilized by plant uptake as well as a suite of microbial processes, including assimilation, denitrification, and anaerobic ammonium oxidation (Kadlec and Wallace, 2009; Javanaud et al., 2011; Wu et al., 2017). Due to the complex nature of these processes, past assessments of plant uptake in nature-based systems have varied widely, with reported fractions of nitrogen removal attributable to plant uptake spanning three orders of magnitude (i.e., 0.5–90%) (Meers et al., 2008; Saeed and Sun, 2012), though these differences may partly be explained by variations in plant maturation, plant types and nitrogen loading rates.

Rather than attempting to discriminate among mechanisms, researchers often make simplifying assumptions that can lead to large errors when quantifying nitrogen uptake rates. In particular, researchers often assume that all nitrogen in plants is derived from wastewater in these systems (Healy and Cawley, 2002; Geng et al., 2019; Chen et al., 2014; Du et al., 2018). This is a reasonable assumption if other sources of nitrogen (e.g., soil) are absent. However, uptake will be overestimated if wetland plants have access to other nitrogen sources. Differences between nitrogen removal in planted and unplanted (control) wetlands have also been used to estimate plant uptake of nitrogen (Drizo et al., 1997; Kantawanichkul et al., 2009; Paranychiankis et al., 2016), but this comparison ignores the importance of plant roots in stimulating microbial nitrogen removal in the rhizosphere (Zhai et al., 2013). To overcome these limitations, researchers have also quantified plant uptake in bench-scale wetland microcosms using ^{15}N-tracers (Zhang et al., 2016; Messer et al., 2017; Hu et al., 2016), which have also been widely used to characterize microbial nitrogen cycling in natural ecosystems (Song et al., 2013; Salk et al., 2017) and constructed wetlands (Erler et al., 2008; Rambags et al., 2019). However, in pilot-scale to full-scale wetlands, materials costs associated with ^{15}N-tracers alone could be prohibitively expensive.

Natural abundance stable isotope mixing models provide an alternative approach for estimating plant uptake of nitrogen. Stable isotopes have been widely used at natural abundance levels to study biogeochemical processes (Dawson et al., 2002; Fry, 2006) and nitrogen cycling in aquatic ecosystems (McClelland and Valiela, 1998; Cole et al., 2004; Reinhardt et al., 2006; Bannon and Roman, 2008; Kohzu et al., 2008; Søvik and Mørkved, 2007; Erler et al., 2010; Kaushal et al., 2011; Chen et al., 2014). However, despite their versatility, the application of natural abundance stable isotopes to assess nitrogen uptake in constructed wetlands has been limited (Fair and Heikoop, 2006).

In nature-based systems like the horizontal levee, the primary sources of nitrogen are wastewater and soil. With isotope fingerprinting, measurements of nitrogen isotopes in plants can be used to identify their sources of nitrogen, because isotope ratios in plant tissues typically match their source of nitrogen (Craine et al., 2015). Furthermore, wastewater-derived nitrogen is frequently enriched in ^{15}N (δ^{15}N $\approx$ 25–35‰). As a result, its "isotopic signature" (i.e., δ^{15}N or nitrogen isotope ratio) is distinguishable from other nitrogen sources, such as soils (δ^{15}N $\approx$ 0–10‰), the atmosphere (δ^{15}N $\approx$ −15 to +15‰) or nitrogen derived from fixation (δ^{15}N $\approx$ 0‰) (Heaton, 1986; Kendall, 1998). The isotopic signatures, which are commonly termed 'end-members', of these different sources can be used in mixing models to determine the relative contribution of those sources to the nitrogen found in plants.

In this study, we present a new method for quantifying plant uptake of nitrogen in nature-based treatment systems using a stable isotope mixing model in a pilot-scale horizontal levee. We developed this method in a horizontal levee by coupling: (1)

isotopic analysis of plants, wastewater, and soils; (2) remote sensing-derived biomass measurements; and, (3) water quality data. We used this information to assess the contribution of plant uptake to nitrogen removal in this system. This approach is a promising tool for understanding nitrogen fate and managing nitrogen species in both nature-based treatment systems and in natural ecosystems.

2. Materials and methods

2.1. Field site

Nitrogen uptake was studied at a demonstration-scale horizontal levee system at the Oro Loma Sanitary District wastewater treatment plant in San Lorenzo, CA (Cecchetti et al., 2020a). The subsurface wetland contained 12 gently sloped (1:30) parallel treatment cells (1 m deep, 12 m wide and 46 m long) that were hydraulically isolated from each other. The cells were used to test four treatments (i.e., swale-depression cells, wet meadows with fine and coarse topsoil, and riparian willow cells) that varied in terms of their topography, soil type and native plant community type. Underlaying the 60 cm of topsoil were two 15-cm deep layers of sand and gravel that were amended with organic carbon (i.e., wood chips) to promote microbial denitrification.

Influent samples were collected from a pump station that delivered nitrified secondary municipal wastewater effluent into 12 wetland cells approximately 5 cm below the surface at the top of the slope. The effluent from each cell flowed into a gravel trench spanning the end of the cell and was collected in perforated PVC pipes at the bottom of the trench, from where effluent samples were collected.

Native plants, mainly of the families *Cyperaceae* (sedges), *Juncaceae* (rushes) and *Salicaceae* (willows), were planted between September 2015 and February 2016, approximately 15 months before nitrified wastewater effluent was first introduced into the subsurface. Prior to this time, the cells were irrigated with shallow groundwater obtained from a well located approximately 50 m from the wetland.

Additional details regarding the hydraulics, design, and wetland treatments, including the plant species and the number of individuals of each species planted in the wetland, are available elsewhere (Cecchetti et al., 2020a).

2.2. Solid sample collection and processing

δ^{15}N and δ^{34}S were measured in foliar and soil samples before and after wastewater effluent was introduced into the horizontal levee to assess isotopic discrimination during uptake (prior to wastewater introduction) and as inputs for source contribution models (after introduction of wastewater) (Fig. 1).

Samples of new growth plant leaves were collected from Baltic rushes (*J. balticus* syn. *J. arcticus*; in cells A-G, I, and K) and Arroyo willows (*S. lasiolepis*; in cells H, J, and L) every 3–6 months between August 2016 and June 2019. These species were chosen due to their dominance in the plant community (i.e., *S. lasiolepis* in willow cells and *J. balticus* in wet meadow cells). The δ^{15}N values for these samples were indistinguishable (p = 0.71; Wilcoxon signed-rank test) from composite biomass samples, as in Fig. S1 (*J. balticus*) of the supporting information (SI). Samples were collected at various distances along the wetland slope. In riparian-type cells, foliar samples from *S. lasiolepis* were collected at 7–10 locations along three transects that were parallel to water flow in each cell. A total of 332 foliar samples of *S. lasiolepis* were collected on 8 sampling dates. In all other cells, samples of *J. balticus* were collected and pooled into 7 distance-resolved samples per cell from transects

perpendicular to the direction of flow along the slope. A total of 158 pooled foliar samples of *J. balticus* were collected on 5 sampling dates. Leaves were separated from their petioles at sampling (when appropriate) and placed in paper envelopes. The envelopes were placed inside of plastic bags and stored on ice prior to returning to the lab. Leaf samples were dried at 65 °C for 48 h immediately upon returning to the lab.

Soil samples were collected for isotope analysis from the top 10 cm of the soil. In 2016, these samples were collected from 9 randomly selected locations per cell. Additional soil samples were collected in 2017 and 2018 for comparison. Soil samples were stored on ice prior to returning to the lab. Soil samples were freeze dried upon returning to the lab using a Labconco FreezeZone 12 Freeze Dryer (Labconco, Kansas City, MO).

Dried leaf and soil samples were ground to a fine powder (200 mesh) using a mortar and pestle, a SPEX SamplePrep 8000 Mill (SPEX SamplePrep, Metuchen, NJ) or a Mini-BeadBeater (Biospec Products, Bartlesville, OK). Ground samples were weighed on a Sartorius microbalance (Sartorius Laboratory Instruments, Goettingen, Germany) and packed into tin capsules (Costech Analytical Technologies, Valencia, CA). Capsules were compressed into spheres and stored in 96-well culture plates prior to analysis.

2.3. Water sample collection and processing

Influent and effluent water samples were collected once or twice per month between April 2017 and June 2019 for the measurement of water quality parameters (e.g., pH) and concentrations of anions and cations (e.g., NO_3^-) as described previously (Cecchetti et al., 2020a).

To investigate $\delta^{15}N$ and $\delta^{18}O$ in nitrate spatially, porewater samples from throughout the entire wetland and on transects along the flow path within the first 10 m of the wetland were collected as described previously (Cecchetti et al., 2020a). Briefly, 25−100 mL samples were collected from depths between 0.1 and 0.9 m into syringes using steel sediment porewater samplers and were filtered directly through 0.2-μm nylon syringe filters into 60-mL or 120-mL polypropylene Nalgene bottles (Sigma-Aldrich, St. Louis, MO, USA). Samples were stored on ice for transport and frozen within 4 h.

Prior to isotopic analysis, samples were processed according to the procedure detailed by Granger and Sigman (2009) for removing nitrite to limit interferences during nitrate isotope measurements. After thawing, 10−30 mL subsamples were transferred to acid-washed 60-mL HDPE bottles. 20 μL per mL sample of a 5% w/v sulfamic acid solution in 5% v/v HCl were added to convert nitrous acid to N_2 gas. Samples were sealed and left on a shake table at ambient temperature or 30 °C for 20 min before being adjusted back to a circumneutral pH with 10 μL of a 2M NaOH solution per mL sample. Nitrate concentrations in processed samples were quantified using ion chromatography. Processed samples were refrozen prior to being shipped on ice to the analytical facility.

2.4. Analytical methods

The stable isotope composition of soil samples and plant tissue samples was determined according to previously described methods (Mambelli et al., 2016). Briefly, $\delta^{15}N$ and $\delta^{34}S$ (as well as % N and %S) were determined by continuous flow (CF) triple isotope analysis using a CHNOS Elemental Analyzer (vario ISOTOPE cube, Elementar, Hanau, Germany) interfaced in line with a gas isotope ratio mass spectrometer (IRMS) (IsoPrime 100, Isoprime Ltd, Cheadle, UK). Isotope abundances are presented in δ notation as deviations from standard references (atmospheric nitrogen (AIR) and Vienna Canyon Diablo Troilite (VCDT) for $\delta^{15}N$ and $\delta^{34}S$, respectively) in parts per thousand (‰). Long-term precision for

$\delta^{15}N$ and $\delta^{34}S$ determinations was ±0.20‰ and ±0.40‰. Additional characterizations of the collected soil samples (e.g., soil phosphorus content) are described elsewhere (Cecchetti et al., *in prep*).

Concentrations of anions, cations, organic carbon and total nitrogen were measured in water samples according to standard methods (APHA, 2012), as previously described (Cecchetti et al., 2020a). Briefly, nitrate and other anions (e.g., Cl^-, NO_2^-, and SO_4^{2-}) were measured by ion chromatography with an IonPac AS23 column according to U.S. EPA Method 300.0. Cations were measured by ion chromatography as previously described (Thomas et al., 2002). Non-purgeable organic carbon (NPOC) and total nitrogen (TN) were measured on a Shimadzu TOC-V/CSH analyzer with an attached TN-1 unit according to standard methods (Method 5310B; APHA, 2012).

Measurements of $\delta^{15}N$ and $\delta^{18}O$ in nitrate were made with the bacterial denitrification assay (Sigman et al., 2001) on a Thermo Finnigan GasBench + PreCon trace gas concentration system interfaced to a Thermo Scientific Delta V Plus isotope ratio mass spectrometer (Thermo Electron GmbH, Bremen, Germany). Samples were purged through a double-needle sampler into a helium carrier stream (25 mL min^{-1}). Gas samples passed through a CO_2 scrubber (Ascarite) and N_2O was concentrated in two liquid nitrogen cryo-traps. N_2O was carried by helium to the IRMS via an Agilent GS-Q capillary column (30 m × 0.32 mm, 40 °C, 1.0 mL min^{-1}). Provisional isotope ratios were adjusted to final values using NIST-certified calibration standards USGS 32, USGS 34 and USGS 35 (National Institute of Standards and Technology, Gaithersburg, MD, USA) and are presented in δ notation as deviations from standard references (atmospheric nitrogen (AIR) and Vienna Standard Mean Ocean Water (SMOW) for $\delta^{15}N$ and $\delta^{18}O$, respectively). The precision for $\delta^{15}N$ and $\delta^{18}O$ measurements was ±0.4‰ and ±0.5‰, respectively.

2.5. Plant nitrogen source contribution models

We used mixing models to calculate the fraction of nitrogen in plant biomass derived from soil and wastewater nitrogen, respectively. Of mixing model types available, linear and Bayesian mixing models each have their own set of strengths and weaknesses (see section S1 of the SI). We developed both linear and Bayesian mixing models (Evaristo et al., 2017) to test the utility and flexibility of these two methods for calculating plant uptake of nitrogen. Soil nitrogen and wastewater-derived nitrate were the main sources of nitrogen available to wetland plants. Contributions of nitrogen from atmospheric deposition (0.1% of total nitrogen inputs) and fixation of N_2 by wetland plants (0.5% of total nitrogen inputs) were considered negligible based on upper bound estimates found in the literature for similar locations (Schwede and Lear, 2014; NADP, 2019) or natural wetland systems (Bowden, 1987). Discrimination between nitrogen isotopes during plant uptake was assumed to be negligible based on similarities between $\delta^{15}N$ in baseline soil and plant tissue samples (e.g., samples collected prior to the introduction of wastewater to the subsurface in April 2017; Fig. 2), which was consistent with past findings (Craine et al., 2015). Mixing model assumptions are discussed in section S1 of the SI.

A two-source Bayesian mixing model was developed using MixSIAR (R package, Stock and Semmens, 2013) with three factors: subsurface concentrations of wastewater-derived nitrate (continuous), distance of sampling locations along the slope (continuous), and wetland cell (random). Soil and wastewater nitrogen were input as model end-members. A Dirichlet distribution was used as the prior distribution in all Bayesian mixing models.

A two-source linear mixing model was applied to our data according to Eqs. (1) and (2):

$$\delta^{15}N_{Plants} = \left(\delta^{15}N_{Soil}\right)(f_{Soil}) + \left(\delta^{15}N_{ww}\right)(f_{ww}) \qquad (1)$$

$$1 = f_{Soil} + f_{ww} \qquad (2)$$

where $\delta^{15}N_{Plants}$, $\delta^{15}N_{Soil}$, and $\delta^{15}N_{ww}$ are the nitrogen isotope signatures of plants, soil and wastewater nitrogen. The fractional contributions of soil nitrogen and wastewater nitrogen to plants are represented by f_{Soil} and f_{ww}, respectively. The linear mixing model ultimately used to calculate plant uptake in this study underwent multiple iterations that are described in section S1 of the SI. In the final iteration of the mixing model (section S1.2.3), we used end-member values as described in section S4 of the SI. For $\delta^{15}N_{Soil}$, we used average $\delta^{15}N$ values for soil-derived porewater nitrate (section S4.3) and for $\delta^{15}N_{ww}$, we used spatially-resolved changes in the $\delta^{15}N$ of wastewater-derived nitrate in porewater (section S4.2). This model was applied to individual plant samples, allowing us to calculate means, medians and confidence intervals for f_{Soil} and f_{ww} by cell, cell type and distance along the slope (Phillips and Gregg, 2001).

2.6. Biomass measurements

Above-ground biomass was estimated using a regression developed from temporally resolved total biomass samples and high resolution 4-band remote sensing imagery (Planet Team, 2017). Over 40 geolocated biomass samples were collected by harvesting all living plant biomass above the ground surface in 0.25 m^2 quadrats. On two dates, dead biomass was collected to facilitate estimates of turnover rates (section S2.6 of the SI). Biomass samples were stored in plastic bags on ice before returning to the laboratory where they were weighed before and after being dried at 65 °C for at least 48 h. The difference in weight was used to calculate moisture content.

On each biomass sampling date, we used remote sensing data to compute three vegetation indices: normalized difference vegetation index (NDVI), green normalized difference vegetation index (GNDVI), and simple ratio (SR) as described in section S2 of the SI. We used these vegetation indices to develop three separate regressions between vegetation index and biomass. The SR-based regression was selected for further analyses because it explained the most variance and had the smallest error. Dry weights of below-ground plant biomass (primarily composed of roots) were calculated using relationships between mean annual temperature and below-ground biomass reported in the literature (Gill et al., 2002).

To calculate total above-ground and below-ground biomass production (i.e., net primary production), peak live biomass measurements were multiplied by biomass turnover rates (separately for above-ground and below-ground biomass). Details are provided in section S2 of the SI.

2.7. Plant uptake calculations

Plant uptake of wastewater-derived nitrogen was quantified by multiplying the total production of above-ground and below-ground biomass over time by: (1) the elemental composition of biomass (%N), to determine the total amount of nitrogen stored in plant biomass; and, (2) the fraction of biomass nitrogen derived from wastewater (f_{ww}) from the linear mixing model, to determine the mass of that nitrogen obtained from wastewater. These calculations were performed on individual plant samples to produce spatial distributions of plant uptake (total and from wastewater) over the wetland slope. Spatial distributions were then integrated over the length of the wetland in each cell to produce weighted average values of plant uptake over the entire wetland slope for each cell.

2.8. Statistical analyses

Statistical analyses were performed in Excel (Microsoft Corporation, Redmond, WA, USA) using the Real Statistics Resource Pack software (Release 5.4; Zaiontz, 2018). The collected data typically were not distributed normally and reported p-values were therefore derived from non-parametric analyses (i.e., Wilcoxon signed-rank test for paired samples and Mann-Whitney tests for independent samples) unless otherwise specified. For data that were not normally distributed, reported values represent the median with confidence intervals presented according to: (95% CI = lower confidence interval, upper confidence interval), unless specified otherwise. When data were normally distributed (i.e., with a p-value > 0.05 for both Shapiro-Wilk and d'Agostino-Pearson tests), parametric analyses (e.g., t tests) were used as specified in the text. For normally distributed data, reported values represent the mean ± the standard deviation of the mean.

3. Results and discussion

Over the two-year monitoring period (6/2017−6/2019), 3570 kg of nitrogen were applied to the horizontal levee. The 1660 kg of nitrogen that left the system in wetland effluent was mainly due to overland flow (Fig. 1). These values were calculated based on aqueous nitrogen concentrations and average flow rates reported previously (Cecchetti et al., 2020a) as summarized in the dataset on Mendeley Data (Cecchetti et al., 2020b). As described by Cecchetti et al. (2020a) there were three operational phases during this period (6/2017−11/2017, 11/2017−7/2018, and 7/2018−6/2019), in which flow within the horizontal levee was progressively adjusted to improve system performance. Nitrogen removal increased from 48% of the influent nitrogen mass during the first two monitoring phases to 62% in the final monitoring phase, with >96% removal of nitrogen in the 9 cells operated without overland flow during the final monitoring phase (Cecchetti et al., 2020a). When overland flow was eliminated, nitrogen removal in the horizontal levee was significantly more efficient than has typically been reported for other constructed wetland systems (Cecchetti et al., 2020a; Kadlec
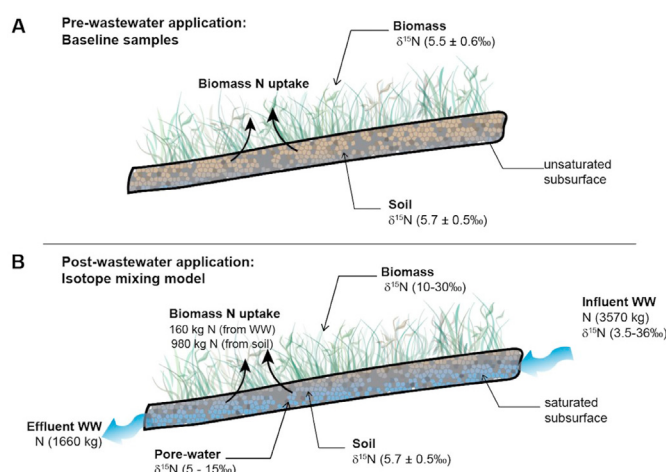
Fig. 1. Nitrogen mass and isotope flows that were used to evaluate plant uptake of nitrogen. Panel A represents baseline samples, which were collected before the application of wastewater effluent. Panel B represents the isotope mixing model used in this study (post-application). Ranges of $\delta^{15}N$ for porewater, biomass, and influent wastewater in Panel B are approximate. Acronyms: WW = wastewater.

and Wallace, 2009).

Approximately 81% of the applied nitrogen consisted of nitrate. The remainder was comprised of dissolved organic nitrogen (7%), nitrite (3%) and ammonium (9%). Therefore, the most significant nitrogen removal processes in this system were those related to nitrate removal (i.e., microbial assimilation, denitrification, and plant uptake). The full dataset can be found on Mendeley Data (Cecchetti et al., 2020b).

3.1. Nitrogen isotope signatures of plants

Data collected throughout the monitoring period were consistent with previous findings that nitrogen in municipal wastewater tends to be enriched isotopically relative to other sources (Heaton, 1986; Kendall, 1998). $\delta^{15}N$ values measured in wastewater had a median value of 9.8‰ (ranging from 3.5‰ to 35.9‰) compared to $\delta^{15}N$ values of 5.7 ± 0.5‰ observed in soil samples.

Prior to introduction of wastewater into the subsurface in April 2017 (Fig. 1a), new-growth foliar plant samples were statistically indistinguishable from soil in terms of $\delta^{15}N$ ($p = 0.59$ and 0.41 from two-tailed t tests for *J. balticus* and *S. lasiolepis*) and also in terms of $\delta^{34}S$ for *J. balticus* ($p = 0.12$) (Fig. 2), which was consistent with past findings (Tcherkez and Tea, 2013; Craine et al., 2015). In terms of $\delta^{34}S$, *S. lasiolepis* was only offset by < 1‰ ($p < 0.01$) from the soil.

As expected, after wastewater effluent was introduced into the subsurface in April 2017 (Fig. 1b), shifts in the values of $\delta^{15}N$ were observed in foliar samples (Fig. 2). After April 2017, $\delta^{15}N$ in foliar samples (of all types) had a median value of 13.8‰ with 95% of measured values falling between 7.4‰ and 23.7‰. This sample distribution was significantly higher ($p < 0.001$) than $\delta^{15}N$ values in plants and soils prior to the introduction of wastewater, despite large variations among individual measurements. More details on the distributions of $\delta^{15}N$ values from different sample types are included in section S3 of the SI and Fig. S6 $\delta^{34}S$ measured in *J. balticus* did not change significantly ($p = 0.51$) after wastewater was introduced, though $\delta^{34}S$ values in *S. lasiolepis* increased significantly ($p < 0.001$), suggesting these plants were accessing different sources of sulfur.

3.2. Mixing models

Despite the advantages associated with Bayesian mixing models (section S1.1.2 of the SI; Phillips et al., 2014), we used a linear mixing model to calculate plant uptake of wastewater-derived nitrogen because it was computationally simpler, could be applied flexibly to individual data points and avoided bias observed in Bayesian models (section S1.2 of the SI) during method development. We progressively optimized our linear mixing models to reflect the conditions at the horizontal levee field site more accurately as described in section S1 of the SI.

3.2.1. Endmember values

Throughout the monitoring period, soil nitrogen values were normally distributed with average $\delta^{15}N$ values of 5.7 ± 0.5‰ and a range from 3.8‰ to 6.8‰ (section S4 of the SI). Conversely, there were wide variations in $\delta^{15}N$ values in wastewater nitrate applied to the system, which were log-normally distributed with a median value of 9.8‰ and a range from 3.5‰ to 35.9‰. Histograms of soil and wastewater $\delta^{15}N$ are presented in Fig. S6. Although there was some overlap, differences in $\delta^{15}N$ from the two sources were significant ($p < 0.001$). The range of observed values for wastewater-derived nitrogen was also consistent with previous findings (Kendall, 1998). Measurements that fell outside of the range of previously reported values likely were caused by operational conditions occurring during the first 6 months of the monitoring period, when influent passed through a surface flow wetland prior to entering the subsurface (Cecchetti et al., 2020a). In the surface flow wetland, nitrate was partially denitrified, and the residual nitrate pool became enriched in ^{15}N (section S4.1 of the SI).

We also observed progressive enrichment of $\delta^{15}N$ and $\delta^{18}O$ values as nitrate was removed in the subsurface. This phenomenon was consistent with the results of past studies of denitrification (Kendall, 1998; Kendall et al., 2007), suggesting that denitrification was important in the subsurface. Specifically, values of $\delta^{15}N$ and $\delta^{18}O$ increased according to a tightly coupled trend as concentrations of nitrate decreased (section S4.2 of the SI). Additionally, enrichment factors (section S4.2.1 of the SI) for both $\delta^{15}N$ and $\delta^{18}O$ were consistent with past research on denitrification in subsurface and groundwater systems (Böttcher et al., 1990; Aravena and Robertson, 1998; Mengis et al., 1999). Enrichment of nitrate in ^{15}N due to denitrification likely explains why some foliar $\delta^{15}N$ values were higher than the values of $\delta^{15}N$ measured in wastewater-nitrate collected on preceding sampling dates, which should have reflected the wastewater nitrogen supplied to those foliar samples (section S3.2 of the SI). Progressive depletion of nitrate and enrichment in ^{15}N was observed consistently across wetland cell types and all four dates when porewater samples were collected
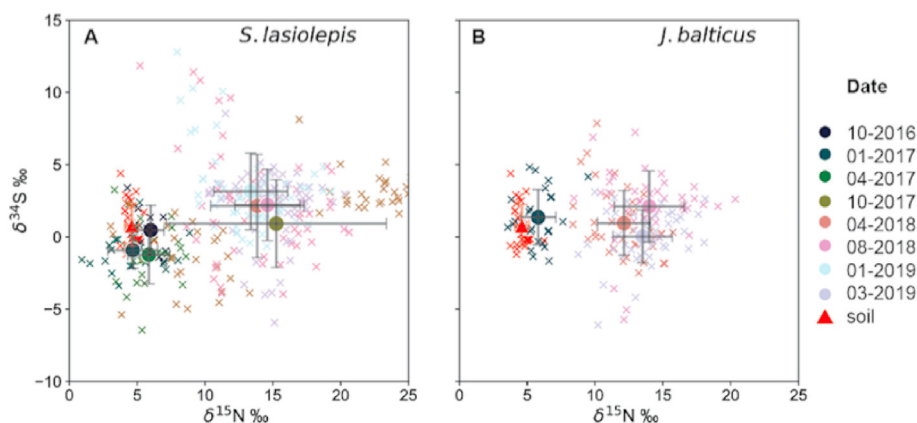
Fig. 2. $\delta^{15}N$ and $\delta^{34}S$ in new-growth foliar samples from (a) Arroyo willows (*S. lasiolepis*) collected in cells H, J and L and (b) Baltic rushes (*J. balticus*) collected in cells A-G, I and K. Darker symbols corresponded to dates (10−2016 through 04−2017) prior to the application of wastewater to the system. Error bars represent the standard deviation of collected samples.

(Figures S4.1 and S4.2).

Within the subsurface, wastewater-derived nitrate was denitrified in the first 5 m of the slope. Changes in concentrations of wastewater nitrate in this region were best approximated by a zero-order removal process. Fitted zero-order equations were used to model nitrate concentrations at distances less than 5 m and were paired with enrichment factors (section S4.2.1 of the SI) to model spatially-resolved wastewater endmember values that could be used to apply the linear mixing model to individual plant samples (section S4 of the SI).

At distances beyond 5 m, porewater nitrate concentrations were below 0.4 mg N L^{-1} and did not exhibit significant trends (Fig. S8e). The coupling of $\delta^{15}N$ and $\delta^{18}O$ values observed in the majority (approximately 75%) of samples collected within the first 5 m was not observed in other parts of the wetland: ratios of $\delta^{18}O$ to $\delta^{15}N$ were significantly (p < 0.001) higher ($\delta^{18}O/\delta^{15}N = 1.8$) relative to the first 5 m of the slope ($\delta^{18}O/\delta^{15}N = 0.8$) (Fig. S12). When porewater nitrate $\delta^{15}N$ and $\delta^{18}O$ were plotted in dual isotope space (i.e. $\delta^{18}O$ v. $\delta^{15}N$; Fig. S13), values from these two parts of the wetland clustered in distinct regions, suggesting the presence of two sources of nitrate in the porewater.

Porewater nitrate with this second isotopic signature (i.e., higher $\delta^{18}O/\delta^{15}N$ ratios) were considered to be attributable to a separate source of nitrate from wastewater-derived nitrate and were used as the second endmember in the mixing model as described in section S1 of the SI. Similar to wastewater-derived nitrate, we observed increasing $\delta^{18}O$ and $\delta^{15}N$ values as nitrate concentrations decreased within this nitrate pool. However, significant spatial trends were not observed, suggesting formation of nitrate from this source occurred across the entire wetland ($r^2 < 0.01$ for all regression types tested; Fig. S14). Based on their significantly greater $\delta^{18}O$ values, we hypothesized that this second source of nitrate was derived from soil nitrogen and/or decay of plant biomass (see section S4.3 of the SI for additional information).

We used an adapted version of a Keeling plot (Pataki et al., 2003) to identify the source isotope signature of this second nitrate pool, which was approximately 3.2‰ (Fig. S15). This value was offset by approximately 2‰ from values of $\delta^{15}N$ measured in soil, which was consistent with the expectation that processes converting soil nitrogen to nitrate (i.e., ammonification and nitrification) will discriminate against heavier isotopes. For example, fractionation of -1.4 to -2.3‰ was observed during mineralization of organic nitrogen in saturated sediments (Möbius, 2013). Additionally, this source of nitrate was only observed after wastewater was introduced to the subsurface, which then became saturated with water (Cecchetti et al., 2020a). Prior to that point, the wetland was only periodically irrigated with well water. Saturated conditions were favorable to denitrifiers that consumed nitrate and enriched the $\delta^{15}N$ values in the residual soil nitrate pool available to plants. This may explain our observation that even plants located in portions of the wetland without contact with wastewater-derived nitrate (i.e., plants located more than 5 m from the inlet to the wetland in cells without overland flow) became enriched in $\delta^{15}N$ after introduction of wastewater. The signal for these plants tended to match this second isotopic signature.

Alternatively, because the first year of monitoring included extended periods of substantial overland flow (e.g., 40–80% of applied wastewater flowed over the wetland surface; Cecchetti et al., 2020a), it is also possible that a fraction of the wastewater-derived nitrogen was taken up into plant biomass at distances beyond 5 m and then cycled back into the subsurface through decomposition of organic matter. If so, this would further justify the use of this second nitrate pool as an endmember, as opposed to solid-phase soil $\delta^{15}N$ values, to avoid inflated plant uptake estimates in subsequent years caused by retention of enriched wastewater nitrogen through internal nitrogen cycling.

3.2.2. Mixing model results

According to the model predictions, throughout the monitoring period 14% of the nitrogen incorporated into plant biomass came from wastewater. The remaining 86% was most likely derived from the soil, which was rich in nitrogen, phosphorus and organic carbon (Cecchetti et al., in prep). The fraction of plant nitrogen coming from wastewater varied with distance along the wetland slope. Approximately 81% of plant nitrogen was derived from wastewater within the first 2 m of the wetland, which was significantly more (p < 0.001) than the 13% that was derived from wastewater in the last two-thirds of the wetland (Fig. 3). There were no significant differences in the fraction of biomass nitrogen coming from wastewater among cells with different planting regimes (p = 0.16) or sediment types (p = 0.48) and no seasonal trends in the fraction of nitrogen uptake from wastewater, though this may have been partly due to the temporal resolution of sampling (Fig. S16).

We estimated that a low but measurable fraction of plant nitrogen came from wastewater at distances beyond 5 m (approximately 5–10% for wet meadow cells and 10–20% for willow cells) despite the fact that wastewater nitrogen was not detected in porewater beyond the first 10 m of the cells. It is possible that this was caused by: (1) intermittent periods of overland flow that supplied enriched wastewater nitrogen farther along the slope; or, (2) internal cycling of wastewater nitrogen that had been taken up into biomass beyond 5 m during early parts of the monitoring period.

For these reasons, it is essential to characterize end-member values continuously when applying mixing models to plant uptake calculations in multi-year studies. Because mixing model results were sensitive to small changes in endmember values (e.g., a change of 2‰ in the soil endmember isotope value shifted average mixing model outputs by 25%), using direct measurements of $\delta^{15}N$ from soil (5.7‰), as opposed to the $\delta^{15}N$ from the soil nitrate source in porewater (10.6‰), would have nearly doubled the estimated mass of wastewater nitrogen taken up by biomass (i.e., 300 kg N v. 150 kg N). A detailed comparison of mixing model results, including sensitivity to endmember values and biomass measurements, is provided in section S1.

3.3. Plant uptake measurements

Total above-ground biomass in the wetland varied among wetland cells and increased throughout the monitoring period. The median dry weight (DW) of above-ground standing biomass was 0.7 kg DW m^{-2} (95% CI = 0.3 kg DW m^{-2}, 1.7 kg DW m^{-2}) for the entire site. The median annual peak above-ground biomass, which typically occurred between the middle of May and late July, was 0.7 kg DW m^{-2} throughout the monitoring period, ranging from 0.5 ± 0.1 kg DW m^{-2} in 2017 to 1.5 ± 0.3 kg DW m^{-2} in 2019 – a significant increase (p < 0.001). Below-ground biomass also increased throughout the monitoring period (Gill et al., 2002), from 0.5 ± 0.1 kg DW m^{-2} in 2017 to 1.0 ± 0.1 kg DW m^{-2} in 2019. Below-ground biomass values modeled in this study, as well as distributions between above-ground and below-ground biomass in the horizontal levee, were consistent with past studies of plants at constructed wetlands (Kadlec and Wallace, 2009), where below-ground biomass was often similar to or less than above-ground biomass.

By the end of the monitoring period (June 2019), the peak above-ground standing biomass (i.e., the maximum amount of standing biomass observed annually) was significantly (p < 0.03) greater in cells planted with willows (i.e., 1.8 ± 0.1 kg DW m^{-2}) than
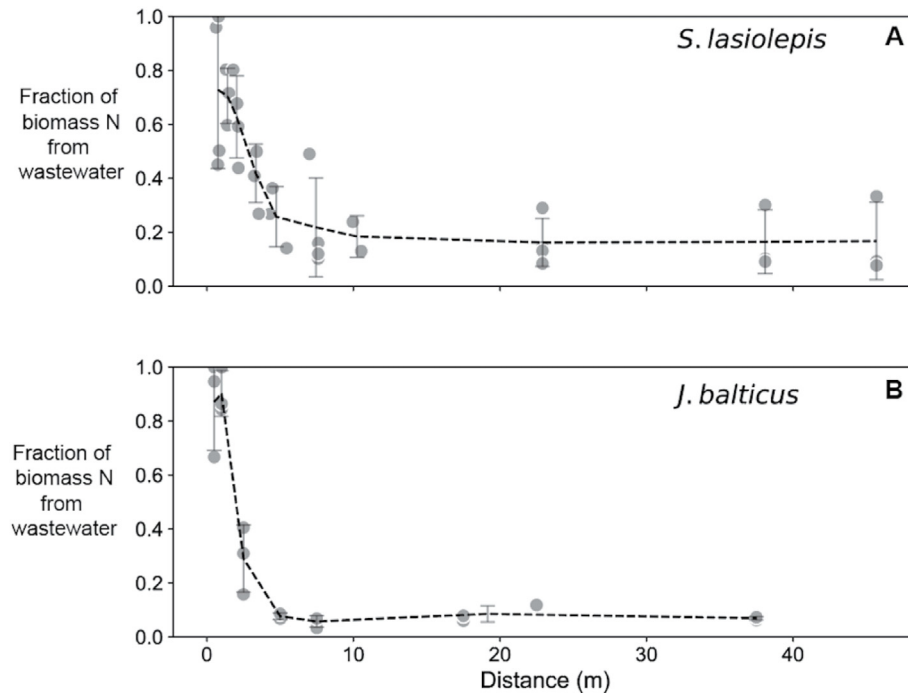
Fig. 3. The fraction of biomass nitrogen derived from wastewater in (a) wet meadow (*J. balticus*) (n = 9) cells and (b) willow (*S. lasiolepis*) (n = 3) cells. Error bars represent one standard deviation across the monitoring dates when foliar samples were collected. The dotted lines represent moving averages.

wet meadow cells (i.e., 1.4 ± 0.2 kg DW m^{-2}). However, due to significantly higher turnover rates in wet meadow cells, the annual above-ground net primary production (i.e., total mass of plant biomass produced annually) was significantly lower (p < 0.01) in cells planted with willows, e.g., 2.0 ± 0.1 kg DW m^{-2} yr^{-1} on average versus 5.7 ± 1.0 kg DW m^{-2} yr^{-1} in those cells planted with wet meadows. This has important implications with respect to the amount of nitrogen incorporated into plant biomass. Our sensitivity analyses revealed that even small changes in average standing biomass can have a significant impact on the amount of nitrogen taken up by plants, particularly in species with high biomass turnover rates. A greater than 10% increase in the modeled mass of nitrogen taken up into plant biomass was observed on average when peak standing biomass increased by 0.1 kg DW m^{-2} over the range of observed biomass values (i.e., 0.3−2.0 kg DW m^{-2}).

Using peak annual biomass measurements (B_{peak} in kg DW m^{-2}; section S2.7 of the SI) and turnover rates (θ_b in yr^{-1}; section S2.6 of the SI), the total mass of nitrogen taken up into plant biomass was calculated according to the equation: $N_{uptake} = B_{peak}\theta_b f_N$. Over the two year monitoring period, the mass fraction of nitrogen in biomass (f_N; kg N (kg DW)$^{-1}$) had median values of 4.0%, 2.2%, and 2.8% for willows (*S. lasiolepis*), Baltic rush (*J. balticus*) and composite samples from wet meadow cells, respectively. This corresponded to a total mass of nitrogen taken up into plant biomass of 1100 kg N (95% CI = 340 kg N, 2400 kg N) over the monitoring period. Based on the results of the mixing model, this yielded a mass of 150 kg N (95% CI = 50 kg N, 330 kg N) of wastewater-derived nitrate removed via plant uptake over the monitoring period, which corresponded to 10% (95% CI = 3%, 22%) of the nitrate and 8% (95% CI = 2%, 17%) of total nitrogen removed from wastewater during the two-year monitoring period. More than a third of wastewater-derived nitrogen taken up by plants (e.g., 51 kg N or 34%) went into willows, which covered close to a quarter of the site.

Plant uptake calculated using this new method differed significantly from estimates made using the approaches applied in previous studies. For example, if all of the nitrogen stored in plant biomass were assumed to be derived from wastewater in the horizontal levee, plant uptake would have accounted for nearly 60% of the removal of applied wastewater nitrogen, rather than the roughly 8% removal calculated using the new methods outlined here. Conversely, if increasing fractional nitrogen content (%N) of plant biomass were assumed to be the derived from wastewater, we would have significantly overestimated uptake of nitrogen from wastewater by willows (e.g., 150 kg N v. the 60 kg N calculated by our method), while underestimating uptake of nitrogen by other plants (e.g., 0 kg N, due to insignificant changes in the %N of composite biomass samples during the monitoring period, v. 90 kg N by our method), despite yielding a similar estimate of uptake over the entire pilot system (e.g., 8%).

The amount of wastewater-derived nitrogen taken up by plants only represented a fraction of their total nitrogen requirements. Of the 1100 kg N taken up by plants during the monitoring period, 980 kg N came from the soil. On the basis of the nitrogen content of the soil at the start of this study (i.e., approximately 0.15% of the soil, or 10,000 kg N) and measured nitrogen removal rates, we estimate that this rate of extraction would only be sustainable for approximately 10 years assuming that all of the soil nitrogen was accessible and none of the plant biomass nitrogen was returned to the soil. However, a large fraction of the nitrogen taken up into plants was deposited as residues annually. For example, although above-ground standing biomass in the wet meadow cells peaked on average at 1.4 kg DW m^{-2} in 2019, the total biomass produced during that year was 6.2 kg DW m^{-2}. Roughly 80% of the produced biomass deposited onto the wetland surface or was consumed by organisms. If the nitrogen stored within those residues was released through decomposition and became available to plants to support subsequent growth, soils could supply sufficient nitrogen for plant growth for nearly 120 years without nitrogen inputs from other sources. This is more consistent with the nitrogen budgets of natural wetlands, in which internal cycling frequently satisfies the majority plant nitrogen demands (Bowden, 1987; Bowden et al., 1991).

3.3.1. Variations in plant uptake rates

The amount of wastewater-derived nitrate removed through plant uptake increased throughout the monitoring period, varied seasonally and among cell types, and decreased with distance along the wetland. Uptake rates were on average 29% higher in the second year of monitoring (6/2018−6/2019) compared to the first (6/2017−6/2018). For example, average uptake rates per cell were 30 g N d^{-1} (8% of applied nitrogen) in the spring of 2018 (3/2018−6/2018) versus 46 g N d^{-1} in the spring of 2019 (3/2019−6/2019). Moreover, the fraction of wastewater N removed by plant uptake was nearly three times higher in the spring of 2019 (23% of removal) than in the preceding spring (8% of removal). This was partly due to lower mass loading rates of wastewater nitrogen in 2019 (which were 50% lower than the preceding year) and also due to higher biomass production, which more than doubled between 2017 and 2019 as plants matured. There were also clear seasonal trends in both plant uptake and the fraction of wastewater nitrogen removed by uptake (Fig. 4), which were both highest in the spring when plant growth was the greatest and lowest in the fall when plants were senescing.

Removal of applied wastewater nitrate via plant uptake per cell was significantly (p < 0.001; one-tailed paired t-test) greater for cells planted with willows (i.e., 23 g N d^{-1}) than for cells dominanted by wet weadow (i.e., 13 g N d^{-1}; Fig. 4b), despite lower primary production rates in willow cells. The extensive rooting zones of willows likely gave them greater access than other plants to wastewater nitrate that flowed primarily in the deeper subsurface layers (Cecchetti et al., 2020a). Additionally, as mentioned previously, the majority of nitrogen was removed at the beginning of the slope (Cecchetti et al., 2020a). Our mixing model indicated that of the 150 kg of nitrogen removed via plant uptake, 63% (97 kg N) was taken up by plants within the first third (15 m) of the slope, with 45% (70 kg N) removed by plants in the first 2 m alone. These uptake trends were consistent across cells throughout the monitoring period.

3.4. Implications for horizontal levee design

There are a variety of design features that could be incorporated into horizontal levees to optimize both nutrient removal and plant uptake of nitrogen. In the pilot-scale horizontal levee, the majority of applied nitrogen (>96%) was removed at the beginning of the wetland before wastewater effluent came into contact with the plants that occupied the remaining 90% of the levee slope. If cells were constructed to increase contact of wastewater nitrogen with plant roots along the entire horizontal levee, the fraction of wastewater nitrogen taken up by plants could increase substantially. Some of the design features proposed previously for optimizing hydraulics (Cecchetti et al., 2020a), such as using granular media with a higher hydraulic conductivity, could also increase contact of wastewater nitrate with plant roots by spreading nitrate from wastewater throughout the subsurface. However, rapid denitrification rates still might limit plant uptake.

Alternatively, designers could consider using multiple water inlet points with subsurface piping manifolds located at regular intervals along the slope to increase the fraction of wastewater nitrogen taken up by biomass. Despite significant denitrification rates, this design feature would increase average subsurface nitrate concentrations further along the slope, increasing the potential for plant uptake in portions of horizontal levees that would not be exposed to wastewater nitrogen loads in systems with a single inlet. Ideally, these manifolds would be spaced every 10−20 m to achieve the greatest possible contact between wastewater nitrogen and plant roots. Other applications, such as batch application of wastewater or systems relying on percolation via surface application of water may also be useful in increasing plant uptake of effluent nitrogen.

Additionally, past research has shown that willows increase the subsurface flow capacity of horizontal levees, which could increase the mass of solutes removed by uptake (Cecchetti et al., 2020a). When combined with our findings that willows have higher nitrogen uptake rates than other wetland plants, it is clear that willows have a significant capacity to promote nitrogen removal in horizontal levees through multiple mechanisms.

It is important to note that nitrogen uptake into plant biomass constitutes a storage mechanism, rather than a permanent sink. However, it is likely that the release of nitrogen into the subsurface from decaying plant residues will either be: (1) taken up by plants again; or, (2) denitrified by microorganisms using organic carbon
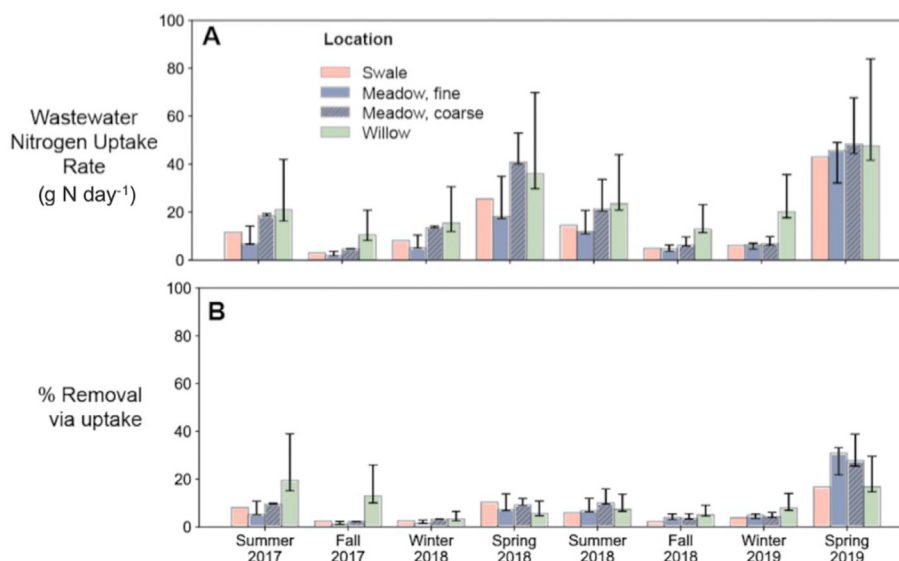

Fig. 4. (a) Uptake rates of wastewater-derived nitrogen into plants in g N d^{-1}, on a per cell basis, and (b) the fraction of wastewater nitrogen removal attributed to plant uptake. Error bars represent 95% confidence intervals (n = 3). Data are segregated by cell type. Error bars are not shown for swale cells due to insufficient data.

from plant residues as an electron donor before nitrogen leaves in the system effluent. For example, if just 10% of the approximately 18,000 kg yr^{-1} of organic carbon from plant biomass decomposed each year — a conservative estimate based on reported litter decomposition rates (Aké-Castillo et al., 2006, review of past studies) — this additional input of organic carbon would be sufficient to denitrify 1600 kg N yr^{-1}, which is greater than annual uptake of nitrogen from all sources in this system and more than 10 times the amount of nitrogen taken up from wastewater alone (150 kg N). A discussion of the relative importance of denitrification and other microbial nitrogen removal mechanisms, as well as the biogeochemical cycling of other redox active species in horizontal levees, is provided elsewhere (Cecchetti et al., *in prep*).

4. Conclusions

In this study, isotope fingerprinting and mixing models were used to evaluate the uptake of nitrogen in a horizontal levee — a new nature-based system for improving water quality which also protects coastal infrastructure from the effects of sea-level rise. Our findings indicate that plant uptake can be responsible for removal of a significant fraction of nitrogen entering the system, with more than 20% of nitrogen removed by plant uptake in the spring of 2019. The exact mechanisms by which the remainder of nitrogen was removed is still unknown. However, on the basis of site biogeochemistry (Cecchetti et al., *in prep*) and isotope fractionation observed in porewater in the first 5 m of the system it appears that a majority of this remaining nitrogen was removed by microbial denitrification.

The new isotope methods detailed in this study are promising techniques for quantifying plant uptake of wastewater-derived nitrogen in natural treatment systems. Mixing models based on isotope fingerprinting provide a more robust means of measuring plant uptake because they do not rely on assumptions that were necessary in previous methods. Isotope methods can improve understanding of nitrogen uptake mechanisms and resolve the wide discrepancies in reported values of nitrogen uptake by plants in other nature-based treatment systems.

More precise estimation of the relative contribution of plant uptake to nitrogen removal is important in multi-benefit treatment systems because it can help designers to prioritize objectives. For example, if plant uptake were found to be insignificant, designers could consider objectives related to habitat creation and planting regimes separately from water quality improvement objectives. Additionally, our results demonstrate that willows have a significant capacity through multiple mechanisms to promote nitrogen removal in horizontal levees. Finally, although plant uptake constitutes a storage mechanism rather than an ultimate removal in systems like the horizontal levee (i.e., where plants are not harvested and removed) labile organic carbon inputs from decaying biomass appear to be sufficient to denitrify the amount of nitrogen reintegrated in the subsurface from senesced biomass.

Author statements

Aidan Cecchetti: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Data Curation, Writing — Original Draft, and Visualization; **Anneliese Sytsma:** Methodology, Validation, Data Curation, Formal analysis, and Writing — Review & Editing; **Angela Stiegler:** Methodology, Investigation, and Writing — Review & Editing; **Todd Dawson:** Conceptualization, Methodology, and Writing — Review & Editing; **David Sedlak:** Conceptualization, Resources, Writing — Review & Editing, Supervision, Project administration, and Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wroa.2020.100070.

References

Aké-Castillo, J.A., Vázquez, G., López-Portillo, J., 2006. Litterfall and decomposition of *Rhizophora mangle* L. in a coastal lagoon in the southern Gulf of Mexico. Hydrobiologia 559, 101–111. https://doi.org/10.1007/s10750-005-0959-x.

American Public Health Association (APHA), 2012. Standard Methods for the Examination of Water and Wastewater, twenty-second ed. American Public Health Association, American Water Works Association, Water Environment Foundation, Washington, DC.

Aravena, R., Robertson, W.D., 1998. Use of multiple isotope tracers to evaluate denitrification in ground water: study of nitrate from a large-flux septic system plume. Ground Water 36 (6), 975–982. https://doi.org/10.1111/j.1745-6584.1998.tb02104.x.

Bannon, R.O., Roman, C.T., 2008. Using stable isotopes to monitor anthropogenic nitrogen inputs to estuaries. Ecol. Appl. 18 (1), 22–30. https://www.jstor.org/stable/40062108.

Böttcher, J., Strebel, O., Voerkelius, S., Schmidt, H.-L., 1990. Using isotope fractionation of nitrate-nitrogen and nitrate-oxygen for evaluation of microbial denitrification in a sandy aquifer. J. Hydrol. 114, 413–424. https://doi.org/10.1016/0022-1694(90)90068-9.

Bowden, W.B., 1987. The biogeochemistry of nitrogen in freshwater wetlands. Biogeochemistry 4 (3), 313–348. https://www.jstor.org/stable/1468671.

Bowden, W.B., Vörösmarty, C.J., Morris, J.T., Peterson, B.J., Hobbie, J.E., Steudler, P.A., Moore III, B., 1991. Transport and processing of nitrogen in a tidal freshwater wetland. Water Resour. Res. 27 (3), 389–408. https://doi.org/10.1029/90WR02614.

Cecchetti, A.R., Stiegler, A.N., Graham, K.E., Sedlak, D.L., 2020a. The horizontal levee: a multi-benefit nature-based treatment system that improves water quality and protects coastal levees from the effects of sea level rise. Water Res. X 7, 100052. https://doi.org/10.1016/j.wroa.2020.100052.

Cecchetti, A., Stiegler, A., Sytsma, A., Gonthier, E., Graham, K., Boehm, A.B., Dawson, T., Sedlak, D., 2020b. Horizontal Levee Monitoring Data. Mendeley Data. https://doi.org/10.17632/xwx83vzmf6.2 vol. 2.

Cecchetti, A.R., Stiegler, A.N., Gonthier, E., Sedlak, D.L., The Fate of Dissolved Nitrogen Species in a Horizontal Levee: Impacts of Electron Acceptors on Seasonal Nitrate Removal Processes. (Manuscript in preparation).

Chen, Y., Wen, Y., Zhou, Q., Vymazal, J., 2014. Effects of plant biomass on nitrogen transformation in subsurface-batch constructed wetlands: a stable isotope and mass balance assessment. Water Res. 63, 158–167. https://doi.org/10.1016/j.watres.2014.06.015.

Cole, M.L., Valiela, I., Kroeger, K.D., Tomasky, G.L., Cebrian, J., Wigand, C., McKinney, R.A., Grady, S.P., da Silva, M.H.C., 2004. Assessment of δ15N isotopic method to indicate anthropogenic eutrophication in aquatic ecosystems. J. Environ. Qual. 33, 124–132. https://doi.org/10.2134/jeq2004.1240.

Craine, J.M., Brookshire, E.N.J., Cramer, M.D., Hasselquist, N.J., Koba, K., Marin-Spiotta, E., Wang, L., 2015. Ecological interpretations of nitrogen isotope ratios of terrestrial plants and soils. Plant Soil 396, 1–26. https://doi.org/10.1007/s11104-015-2542-1.

Dawson, T.E., Mambelli, S., Plamboeck, A.H., Templer, P.H., Tu, K.P., 2002. Stable isotopes in plant ecology. Annu. Rev. Ecol. Systemat. 33, 507–559. https://doi.org/10.1146/annurev.ecolsys.33.020602.09545.

Drizo, A., Frost, C.A., Smith, K.A., Grace, J., 1997. Phosphate and ammonium removal by constructed wetlands with horizontal subsurface flow, using shale as a substrate. Water Sci. Technol. 35 (5), 95–102.

Du, L., Trinh, X., Chen, Q., Wang, C., Wang, H., Xia, X., Zhou, Q., Xu, D., Wu, Z., 2018. Enhancement of microbial nitrogen removal pathway by vegetation in Integrated Vertical-Flow Constructed Wetlands (IVCWs) for treating reclaimed water. Bioresour. Technol. 249, 644–651. https://doi.org/10.1016/j.biortech.2017.10.074.

Erler, D.V., Eyre, B.D., Davison, L., 2008. The contribution of anammox and denitrification to sediment N2 production in a surface flow constructed wetland. Environ. Sci. Technol. 42, 9144–9150. https://doi.org/10.1021/es801175t.

Erler, D.V., Eyre, B.D., Davison, L., 2010. Temporal and spatial variability in the cycling of nitrogen within a constructed wetland: a whole-system stable-isotope-addition experiment. Limnol. Oceanogr. 55 (3), 1172–1187. https://doi.org/10.4319/lo.2010.55.3.1172.

Evaristo, J., McDonnell, J.J., Clemens, J., 2017. Plant source water apportionment using stable isotopes: a comparison of simple linear, two-compartment mixing model approaches. Hydrol. Process. 31 (21), 3750–3758. https://doi.org/10.1002/hyp.11233.

Fair, J.M., Heikoop, J.M., 2006. Stable isotope dynamics of nitrogen sewage effluent uptake in a semi-arid wetland. Environ. Pollut. 140, 500–505. https://doi.org/10.1016/j.envpol.2005.08.005.

Freeman, L.A., Corbett, D.R., Fitzgerald, A.M., Lemley, D.A., Quigg, A., Steppe, C.N., 2019. Impacts of urbanization and development on estuarine ecosystems and water quality. Estuar. Coast 42, 1821–1838. https://doi.org/10.1007/ss12237-019-00597-z.

Fry, B., 2006. Stable Isotope Ecology. Springer Science+Business Media, New York, NY.

Galloway, J.N., Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the nitrogen cycle: recent trends, questions and potential solutions. Science 320, 889–892. https://doi.org/10.1126/science.1136674.

Geng, Y., Ge, Y., Luo, B., Chen, Z., Min, Y., Schmid, B., Gu, B., Chang, J., 2019. Plant diversity increases N removal in constructed wetlands when multiple rather than single N processes are considered. Ecol. Appl. 29 (7), e01965. https://doi.org/10.5061/dryad.80nf679.

Gill, R.A., Kelly, R.H., Parton, W.J., Day, K.A., Jackson, R.B., Morgan, J.A., Scurlock, J.M.O., Tieszen, L.L., Castle, J.V., Ojima, D.S., Zhang, X.S., 2002. Using simple environmental variables to estimate below-ground productivity in grasslands. Global Ecol. Biogeogr. 11, 79–86. https://doi.org/10.1046/j.1466-822X.2001.00267.x.

Granger, J., Sigman, D.M., 2009. Removal of nitrite with sulfamic acid for nitrate N and O isotope analysis with the denitrifier method. Rapid Commun. Mass Spectrom. 23, 3753–3762. https://doi.org/10.1002/rcm.4307.

Harris-Lovett, S., Lienert, J., Sedlak, D.L., 2019. A mixed-methods approach to strategic planning for multi-benefit regional water infrastructure. J. Environ. Manag. 233, 218–237. https://doi.org/10.1016/j.jenvman.2018.11.112.

Healy, M., Cawley, A.M., 2002. Nutrient processing capacity of a constructed wetland in western Ireland. J. Environ. Qual. 31, 1739–1747.

Heaton, T.H.E., 1986. Isotopic studies of nitrogen pollution in the hydrosphere and atmosphere: a review. Chem. Geol. 59, 87–102. https://doi.org/10.1016/0168-9622(86)90059-X.

Hu, Yun, He, Feng, Wang, Long, Sun, Jian, Huang, Tao, Wu, Zhenbin, 2016. The fate of ammonium in integrated vertical-flow constructed wetlands using stable isotope technique. Pol. J. Environ. Stud. 25 (3), 1027–1032. https://doi.org/10.15244/pjoes/61534.

Javanaud, C., Michotey, V., Guasco, S., Garcia, N., Anschutz, P., Canton, M., Bonin, P., 2011. Anaerobic ammonium oxidation mediated by Mn-oxides: from sediment to strain level. Res. Microbiol. 162 (9), 848–857. https://doi.org/10.1016/j.resmic.2011.01.011.

Kadlec, R.H., Wallace, S.D., 2009. Treatment Wetlands, second ed. CRC Press, Boca Raton, FL.

Kantawanichkul, S., Kladprasert, S., Brix, H., 2009. Treatment of high-strength wastewater in tropical vertical flow constructed wetlands planted with *Typha angustifolia* and *Cyperus involucratus*. Ecol. Eng. 35, 238–247. https://doi.org/10.1016/j.ecoleng.2008.06.002.

Kaushal, S.S., Groffman, P.M., Band, L.E., Elliott, E.M., Shields, C.A., Kendall, C., 2011. Tracking nonpoint source nitrogen pollution in human-impacted watersheds. Environ. Sci. Technol. 45 (19), 8225–8232. https://doi.org/10.1021/es200779e.

Kendall, C., 1998. Chapter 16 – tracing nitrogen sources and cycling in catchments. Isotope Tracers in Catchment Hydrology, pp. 519–576. https://doi.org/10.1016/B978-0-444-81546-0.50023-9.

Kendall, C., Elliott, E.M., Wankel, S.D., 2007. Tracing anthropogenic inputs of nitrogen to ecosystems. In: Michener, R., Lajtha, K. (Eds.), Stable Isotopes in Ecology and Environmental Science, second ed. Blackwell Publishing, Malden, MA, USA. https://doi.org/10.1002/9780470691854.ch12.

Kohzu, A., Miyajima, T., Tayasu, I., Yoshimizu, C., Hyodo, F., Matsui, K., Nakano, T., Wada, E., Fujita, N., Nagata, T., 2008. Use of stable nitrogen isotope signatures of riparian macrophytes as an indicator of anthropogenic N inputs to river ecosystems. Environ. Sci. Technol. 42, 7837–7841. https://doi.org/10.1021/es801113k.

Li, X., Bellerby, R., Craft, C., Widney, S.E., 2018. Coastal wetland loss, consequences, and challenges for restoration. Anthropocene Coasts 1, 1–15. https://doi.org/10.1139/anc-2017-0001.

Mambelli, S., Brooks, P.D., Sutka, R., Hughes, S., Finstad, K.M., Nelson, J.P., Dawson, T.E., 2016. High-throughput method for simultaneous quantification of N, C, and S stable isotopes and contents in organics and soils. Rapid Commun. Mass Spectrom. 30 (15) https://doi.org/10.1002/rcm.7605.

McClelland, J.W., Valiela, I., 1998. Linking nitrogen in estuarine producers to land-derived sources. Limnol. Oceanogr. 43 (4), 577–585. https://doi.org/10.4319/lo.1998.43.4.0577.

Meers, E., Tack, F.M.G., Tolpe, I., Michels, E., 2008. Application of a full-scale constructed wetland for treatment of piggery manure: monitoring results. Water Air Soil Pollut. 193, 15–24. https://doi.org/10.1007/s11270-008-9664-5.

Megonigal, J.P., Neubauer, S.C., 2019. Chapter 19: biogeochemistry of tidal freshwater wetlands. Coastal Wetlands, second ed., pp. 641–683. https://doi.org/10.1016/B978-0-444-63893-9.00019-8.

Mengis, M., Schiff, S.L., Harris, M., English, M.C., Aravena, R., Elgood, R.J., MacLean, A., 1999. Multiple geochemical and isotopic approaches for assessing ground water NO3 elimination in a riparian zone. Ground Water 37 (3), 448–457. https://doi.org/10.1111/j.1745-6584.1999.tb01124.x.

Messer, T.L., Burchell, M.R., Böhkle, J.K., Tobias, C.R., 2017. Tracking the fate of nitrate through pulse-flow wetlands: a mesocosm scale 15N enrichment tracer study. Ecol. Eng. 106, 597–608. https://doi.org/10.1016/j.ecoleng.2017.06.016.

Möbius, J., 2013. Isotope fractionation during nitrogen remineralization (ammonification): implications for nitrogen isotope biogeochemistry. Geochem. Cosmochim. Acta 105, 422–432. https://doi.org/10.1016/j.gca.2012.11.048.

National Atmospheric Deposition Program (Nadp; Nrsp-3), 2019. Henry Mall. NADP Program Office, vol. 465. Wisconsin State Laboratory of Hygiene, Madison, WI 53706.

Paranychianakis, N.V., Tsiknia, M., Kalogerakis, N., 2016. Pathways regulating the removal of nitrogen in planted and unplanted subsurface flow constructed wetlands. Water Res. 102, 321–329. https://doi.org/10.1016/j.watres.2016.06.048.

Pataki, D.E., Ehleringer, J.R., Flanagan, L.B., Yakir, D., Bowling, D.R., Still, C.J., Buchmann, N., Kaplan, J.O., Berry, J.A., 2003. The application and interpretation of Keeling plots in terrestrial carbon cycle research. Global Biogeochem. Cycles 17 (1), 1022. https://doi.org/10.1029/2001GB001850.

Phillips, D.L., Gregg, J.W., 2001. Uncertainty in source partitioning using stable isotopes. Oecologia 127, 171–179. https://doi.org/10.1007/s004420000578.

Phillips, D.L., Inger, R., Bearhop, S., Jackson, A.L., Moore, J.W., Parnell, A.C., Semmens, B.X., Ward, E.J., 2014. Best practices for use of stable isotope mixing models in food-web studies. Can. J. Zool. 92 (10), 823–835. https://doi.org/10.1139/cjz-2014-0127.

Planet Team, 2017. Planet application program interface. In: Space for Life on Earth. San Francisco, CA. https://api.planet.com.

Rambags, F., Tanner, C.C., Schipper, L.A., 2019. Denitrification and anammox remove nitrogen in denitrifying bioreactors. Ecol. Eng. 138, 38–45. https://doi.org/10.1016/j.ecoleng.2019.06.022.

Reinhardt, M., Müller, B., Gächter, R., Wehrli, B., 2006. Nitrogen removal in a small constructed wetland: an isotope mass balance approach. Environ. Sci. Technol. 40, 3313–3319. https://doi.org/10.1021/es052393d.

Saeed, T., Sun, G., 2012. A review on nitrogen and organics removal mechanisms in subsurface flow constructed wetlands: dependency on environmental parameters, operating conditions and supporting media. J. Environ. Manag. 112, 429–448. https://doi.org/10.1016/j.jenvman.2012.08.011.

Salk, K.R., Erler, D.V., Eyre, B.D., Carlson-Perret, N., Ostrom, N.E., 2017. Unexpectedly high degree of anammox and DNRA in seagrass sediments: description and application of a revised isotope pairing technique. Geochem. Cosmochim. Acta 211, 64–78. https://doi.org/10.1016/j.gca.2017.05.012.

Schwede, D.B., Lear, G.G., 2014. A novel hybrid approach for estimating total deposition in the United States. Atmos. Environ. 92, 207–220. https://doi.org/10.1016/j.atmosenv.2014.04.008.

Sigman, D.M., Casciotti, K.L., Andreani, M., Barford, C., Galanter, M., Böhlke, J.K., 2001. A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. Anal. Chem. 73 (17), 4145–4153. https://doi.org/10.1021/ac010088e.

Song, G.D., Liu, S.M., Marchant, H., Kuypers, M.M.M., Lavik, G., 2013. Anammox, denitrification and dissimilatory nitrate reduction to ammonium in the East China Sea sediment. Biogeosciences 10, 6851–6864. https://doi.org/10.5194/bg-10-6851-2013.

Søvik, A.K., Mørkved, P.T., 2007. Nitrogen isotope fractionation as a tool for

determining denitrification in constructed wetlands. Water Sci. Technol. 56 (3), 167–173. https://doi.org/10.2166/wst.2007.529.

Stock, B.C., Semmens, B.X., 2013. MixSIAR GUI user manual. Version 3.1. https://github.com/brianstock/MixSIAR. https://doi.org/10.5281/zenodo.56159.

Sutton, M.A., Bleeker, A., 2013. The shape of nitrogen to come. Nature 494, 435–437.

Tcherkez, G., Tea, I., 2013. $^{32}S/^{34}S$ isotope fractionation in plant sulphur metabolism. New Phytol. 200, 44–53. https://doi.org/10.1111/nph.12314.

Thomas, D.H., Rey, M., Jackson, P.E., 2002. Determination of inorganic cations and ammonium in environmental waters by ion chromatography with a high-capacity cation-exchange column. J. Chromatogr. A 956, 181–186. https://doi.org/10.1016/S0021-9673(02)00141-3.

Thornton, J.A., Harding, W.R., Dent, M., Hart, R.C., Lin, H., Rast, C.L., Rast, W., Ryding, S.-O., Slawski, T.M., 2013. Eutrophication as a 'wicked' problem. Lakes Reservoirs Res. Manag. 18, 298–316. https://doi.org/10.1111/lre.12044.

Wu, S., Vymazal, J., Brix, H., 2017. Critical review: biogeochemical networking of iron in constructed wetlands for wastewater treatment. Environ. Sci. Technol. 53, 7930–7944. https://doi.org/10.1021/acs.est.9b00958.

Zaiontz, C., 2018. Real statistics using Excel. www.real-statistics.com.

Zhang, W., Lei, Q., Li, Z., Han, H., 2016. Temporal variation of nitrogen balance within constructed wetlands treating slightly polluted water using a stable nitrogen isotope experiment. Environ. Sci. Pollut. Control Ser. 23, 2677–2683. https://doi.org/10.1007/s11356-015-5485-4.

Zhai, X., Piwpuan, N., Arias, C.A., Headley, T., Brix, H., 2013. Can root exudates from emergent wetland plants fuel denitrification in subsurface flow constructed wetlands? Ecol. Eng. 61P, 555–563. https://doi.org/10.1016/j.ecoleng.2013.02.014.