

# Fate of Dissolved Nitrogen in a Horizontal Levee: Seasonal Fluctuations in Nitrate Removal Processes

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nitrogen removal suggested that autotrophic denitrifiers oxidized Fe(II) sulfides using nitrate as an electron acceptor. These trends were seasonal, with Fe(II) sulfide minerals formed during summer fueling denitrification during the subsequent winter. Overall, around 30% of gaseous nitrogen losses in the winter were attributable to autotrophic denitrifiers. To predict long-term nitrogen removal, we developed an electron-transfer model that accounted for the production and consumption of electron donors. The model indicated that the labile organic carbon released from wood chips may be capable of supporting nitrogen removal from wastewater effluent for several decades with sulfide minerals, decaying vegetation, and root exudates likely sustaining nitrogen removal over a longer timescale.

**KEYWORDS:** denitrification, nature-based treatment, redox, wetlands

activity was lower, strong correlations between sulfate release and

# INTRODUCTION

Humans have significantly transformed the global nitrogen cycle with negative environmental impacts.<sup>1,2</sup> Through the Haber–Bosch process, we have doubled the natural rate of terrestrial N<sub>2</sub> fixation,<sup>3</sup> creating excess nitrogen in agricultural runoff and municipal wastewater effluent that stimulates the growth of toxic algae and causes eutrophication in marine and estuarine systems.<sup>4</sup> Because increasing global temperatures likely will amplify these effects,<sup>5</sup> more effective management of anthropogenic sources of nitrogen is needed.<sup>6</sup>

Municipal wastewater treatment plants are often dominant sources of anthropogenic nitrogen regionally, but engineered nitrogen removal systems require large capital investments for installation<sup>7</sup> and are expensive to operate.<sup>8</sup> In contrast, naturebased treatment systems can remove nitrogen at lower costs while providing other benefits.<sup>9</sup> However, nature-based systems have not been as popular because they often require larger areas and exhibit a diminished performance in cold temperatures.<sup>10</sup>

Horizontal levees are a new type of nature-based treatment wetland that may overcome some of these limitations.

Horizontal levees consist of a wedge of sediments that buttresses storm control levees to protect low-lying urban areas from storm surges<sup>11</sup> while removing contaminants from wastewater effluent in subsurface layers.<sup>12</sup> These systems build upon insights from riparian wetlands<sup>13</sup> and denitrification walls<sup>14,15</sup> and can provide ancillary benefits (e.g., terrestrial habitat, flood control, and recreation) that are valued by local stakeholders.<sup>16</sup> In past studies, horizontal levees removed wastewater-derived contaminants, including nitrogen (primarily as nitrate), in a smaller footprint than other types of constructed wetlands.<sup>12</sup> Additionally, removal of nitrate in these systems remained high even during the winter when the average water temperature decreased from 21 to 17 °C.<sup>12</sup>

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Despite these promising findings, the pathways for nitrogen removal in horizontal levees were not fully characterized in past research. Microbial processes in the subsurface were presumed to be important because isotopic analyses indicated that plant uptake was only responsible for about 10% of wastewater-nitrogen removal.<sup>17</sup> However, the combination of the quantified removal pathways and canonical denitrification did not explain the lack of seasonal variations in dissolved nitrogen removal. Plant senescence and lower water temperatures during winter led to lower nitrogen uptake rates by plants<sup>17</sup> and should have resulted in lower rates of heterotrophic denitrification,<sup>10,18</sup> but overall nitrogen removal rates did not exhibit seasonal variations.<sup>12</sup>

To explain these observations, we hypothesized that the majority of nitrate was removed by microbial denitrification with decreased rates of activity of heterotrophic denitrifier species in winter compensated for by the less temperature-sensitive autotrophic denitrifiers.<sup>19</sup> Autotrophic denitrifiers often reduce nitrate by extracting electrons from sulfide minerals, which can form when the plentiful sulfate found in municipal wastewater effluent is reduced. Our hypothesis was consistent with observations from wetland microcosms<sup>20</sup> and full-scale constructed wetlands<sup>21</sup> where sulfide-driven denitrification was important even when sufficient organic carbon was available for heterotrophic denitrifiers.

In addition to denitrification, other pathways, such as anaerobic ammonium oxidation (anammox), ammonium oxidation coupled to iron reduction (feammox), and dissimilatory nitrate reduction to ammonium (DNRA), may contribute to nitrogen removal in wetlands.<sup>22–24</sup> For example, researchers have indicated that sulfide-driven DNRA can serve as a link between anammox and sulfur cycling in constructed wetlands<sup>25</sup> and tropical wetland sediments.<sup>26</sup>

To better characterize the processes responsible for nitrogen removal in the horizontal levee, we performed mass balances on nitrogen and a suite of redox-active elements (e.g., sulfur and iron) and collected seasonal measurements of redox-active aqueous species. Mass balance approaches have often been used to quantify nitrogen cycle processes in constructed wetlands.<sup>27-31</sup> These methods are advantageous because they (1) can be applied easily at a field scale, (2) do not require specialized instrumentation, and (3) do not involve manipulations that have the potential to bias results.<sup>32</sup> However, mass balances must be paired with additional methods (e.g., <sup>15</sup>N pool dilution studies) to directly quantify nitrogen cycle process rates.<sup>27,32</sup> In our study, mass balance results were supported with measurements of microbial gene abundances<sup>18</sup> and assessments of mineral formation using synchrotron-based X-ray fluorescence spectromicroscopy. Our data were used in a quantitative model of elemental cycling in horizontal levees to predict how subsurface biogeochemistry will impact long-term nitrogen removal.

#### MATERIALS AND METHODS

**Experimental Horizontal Levee Site.** As described in detail previously,<sup>12</sup> the 0.7 ha experimental wetland consisted of 12 hydraulically separated parallel sloped subsurface wetland cells, which were used to test four treatments: (1) cells planted with willows, cells planted as wet meadows with sedges and rushes in (2) coarse, or (3) fine topsoils, and (4) cells constructed with swales. In all cells, coarse or fine clay loam topsoils were underlain by sand and gravel layers. The layers were amended with approximately 24 g wood chips (kg soil)<sup>-1</sup>

(10-30% v/v) on average. The combined depth of the subsurface was 0.9 m. Below the sand and gravel layers, a compacted clay liner prevented infiltration. Effluent from the adjacent conventional activated sludge municipal wastewater treatment plant (https://oroloma.org/sewage-treatment/) was passed through a nitrifying gravel filter prior to being delivered to the subsurface at the top of the slope. A detailed description of the experimental horizontal levee site, including (1) site layouts and design schematics, (2) average effluent composition, (3) descriptions of the wetland treatments, (4) specifications for soil types and wood chips, (5) information on plant species, and (6) a timeline of operational conditions, is available elsewhere.<sup>12</sup>

Sample Collection. Porewater, influent and effluent water, soils, and plant tissues were collected at locations delineated in Figure S1 of the Supporting Information using methods described previously.<sup>12,17</sup> Porewater was collected quarterly along the wetland slope to track changes in chemical indicators. Porewater samples were collected at depths ranging from 0.6 to 0.9 m using stainless-steel PushPoint porewater samplers (MHE Products, East Tawas, MI, USA). Samples were collected from those depths because the majority of the water applied to the system flowed through the sand and gravel sublayers.<sup>12</sup> Porewater samples (n = 259) were collected during multiple seasons. Additionally, over 1000 samples of water flowing into and out of the horizontal levee were collected<sup>12</sup> from the influent pump wet well and the 12 effluent monitoring wells located at the bottom of the wetland slope using a Masterflex E/S portable sampler (Cole-Parmer, Vernon Hills, IL, USA). Water samples were filtered and stored on ice prior to analysis, which normally occurred within 24-48 h.

New growth plant leaves were collected from representative species every 3–6 months between August 2016 and June 2019 across the wetland slope, as described previously.<sup>17</sup> Leaves were placed in plastic bags and stored on ice prior to returning to the lab. Soil samples were collected from the top 10 cm. In 2016, soil samples were collected from nine randomly selected locations per cell. Additional samples were collected in 2017 and 2018 for comparison. In 2018, soil samples were collected at 10 cm intervals at two locations to investigate whether depth stratifications had developed with respect to soil carbon and nitrogen content and whether the overall soil nitrogen content had changed (as described in Section S4.3 of the Supporting Information). Soil samples were stored on ice during transport and frozen prior to processing and analysis.

**Sample Processing and Analysis.** Sample processing and analytical methods were described previously.<sup>12,17</sup> Briefly, anions and cations were analyzed by ion chromatography,<sup>12,33</sup> and non-purgeable organic carbon, dissolved inorganic carbon, and total dissolved nitrogen were measured using a Shimadzu TOC-V/CSH analyzer with an attached TN-1 unit (Shimadzu Scientific Instruments, Columbia, MD).<sup>34</sup> Dissolved metals (i.e.,  $Mn_{(aq)}$  and  $Fe_{(aq)}$ ) were analyzed by inductively coupled plasma mass spectrometry (ICP–MS).<sup>34</sup> Organic nitrogen concentrations were calculated by subtracting concentrations of nitrate, nitrite, and ammonium from total nitrogen.  $\delta^{15}N$  and  $\delta^{18}O$  in nitrate were measured previously<sup>17</sup> by the bacterial denitrification method.<sup>35</sup>

Leaf samples were dried immediately upon returning to the laboratory at 65 °C for 48 h. Soil samples were frozen immediately and then freeze-dried in a Labconco FreezeZone 12 Freeze Dryer (Labconco, Kansas City, MO). Dried leaf and



**Figure 1.** Nitrogen mass balances over the 2 year monitoring period. (a) Distributions of aqueous nitrogen species in the influent, removed nitrogen, and effluent. (b) Mass balances conducted on nitrogen. Blue arrows and the white text represent wastewater-derived nitrogen flows and their magnitude. Net changes in the size of nitrogen storage pools (e.g., "Plant N") are indicated with the black text in the ovals corresponding to each pool. The magnitudes of these changes are represented by  $\pm$ kmol N. Purple arrows, ovals, and text represent the flows of soil nitrogen, soil nitrogen components, and changes in their size. Green, brown, and tan arrows represent the flows of nitrogen from aboveground plant biomass, plant roots, and litter. The figure is not to scale.

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). Soils were analyzed for iron and manganese by inductively coupled plasma atomic emission spectrometry (ICP–AES) after nitric acid/hydrogen peroxide closed vessel microwave digestion. Dried and powdered soil and plant samples were also analyzed for nitrogen, carbon, and sulfur content (% dry weight) and nitrogen, carbon, and sulfur stable isotope ratios ( $\delta^{15}$ N,  $\delta^{13}$ C, and  $\delta^{34}$ S),<sup>36</sup> which are reported elsewhere.<sup>17,37</sup> Isotope abundances for carbon are presented in  $\delta$  notation as deviations from Vienna PeeDee Belemnite (V-PDB) and have a long-term precision of ±0.10‰.

**Mass and Electron Balances.** Mass balances were conducted on nitrogen, organic carbon, sulfur, iron, and manganese<sup>38</sup> according to eqs S1.1 through S1.4, as presented in the Supporting Information. For all elements other than carbon, the sole inputs were the aqueous species in the nitrified secondary effluent wastewater applied to the wetland. Aqueous species left the system only in wetland effluent. Mass inputs and outputs in the aqueous phase were calculated as the product of the aqueous concentration of that species and the flow. As described previously,<sup>17</sup> inputs of nitrogen from atmospheric deposition and N<sub>2</sub> fixation were considered negligible (i.e., each source accounted for less than 0.5% of the total nitrogen inputs). It was also assumed that atmospheric deposition was a negligible input of all other redox-active elements.



Figure 2. Median concentrations with 90th and 10th percentile error bars for nitrate and other dissolved nitrogen species in porewater samples collected from the gravel/coarse sand layer of the subsurface. Note the different scales of the vertial axes. DON = dissolved organic nitrogen.

Inputs of organic carbon from plant biomass were calculated from biomass production<sup>17</sup> and the elemental stoichiometry of the plant biomass as determined previously.<sup>17</sup> Other elements assimilated into plant biomass (i.e., primarily nitrogen and sulfur) were assumed to have come from the soil or from the aqueous phase and were calculated as described previously.<sup>17</sup> Additional details are described in Section S4 of the Supporting Information.

Electron balances were calculated by converting mass loadings of electron acceptors (e.g., nitrate, sulfate) and electron donors (e.g., organic carbon, sulfide) to electron equivalents as described in Section S1.2 of the Supporting Information. Briefly, molar ratios of electron equivalents consumed or produced (eq  $mol^{-1}$ ) were calculated for a standard set of oxidation and reduction half-reactions. Details are described in Section S1.3 of the Supporting Information.

**Mineral Characterization.** Four samples were collected at distances of approximately 1.5, 6, 23, and 38 m from the inlet of the wetland along the centerline of the cell using a 4 in stony soil auger (AMS, Inc., American Falls, ID, USA) in February 2020. Soils were immediately transferred into 50 mL centrifuge tubes, which were filled completely to minimize headspace and stored on ice under a N<sub>2(g)</sub> headspace in an AtmosBag (Sigma-Aldrich, Inc., St. Louis, MO, USA). While still in the AtmosBag, samples were ground with a mortar and pestle before loading into metal sample trays with Kapton tape. Samples were stored frozen under a N<sub>2(g)</sub> headspace prior to analysis.

X-ray fluorescence spectromicroscopy data were collected at the Advanced Light Source (ALS) XFM beamline 10.3.2,<sup>39</sup> Lawrence Berkeley National Laboratory (LBNL), Berkeley, CA, USA, with the storage ring operating in top-off mode at 500 mA, 1.9 GeV. All data were recorded at room temperature in the fluorescence mode using a Si(111) monochromator with the samples oriented at 45° to the incident X-ray beam. Microfocused X-ray fluorescence ( $\mu$ XRF) mapping of iron and sulfur was performed at 7210 eV (100 eV above the Fe K-edge) and 3938 eV (100 eV below the Ca K-edge), respectively, using pixel sizes ranging from 5 × 5 to 12 × 12  $\mu$ m. Iron speciation in sample regions of interest was determined using Fe K-edge X-ray absorption near-edge structure ( $\mu$ XANES) spectroscopy. Further details can be found in Section S5 of the Supporting Information.

**Microbial Community Characterization.** Five samples were collected along the centerline of a coarse wet meadow cell from the gravel sublayer of wetland at distances of approximately 2.5, 6, 15, 23, and 38 m from the inlet in July 2019. Samples were frozen in the field using liquid nitrogen and stored at -80 °C prior to processing. Genomic DNA was extracted from the soil samples, sequenced, and used to quantify gene abundances for microbial processes associated with nitrogen and sulfur cycling in soils. A detailed description of the methods for sample collection, analysis, and data processing is provided in Section S6 of the Supporting Information.

**Statistical Methods.** Statistical analyses were performed in Excel (Microsoft Corporation, Redmond, WA, USA) using the Real Statistics Resource Pack software (Release 5.4).<sup>40</sup> Reported *p* values were derived from non-parametric analyses unless specified. Only when data were normally distributed (i.e., with a *p* value > 0.05 for both Shapiro–Wilk and d'Agostino-Pearson tests) were parametric analyses used.

#### RESULTS AND DISCUSSION

In the pilot-scale horizontal levee, water flow played a critical role in nitrogen removal.<sup>12</sup> Applied wastewater effluent either underwent subsurface flow with residence times of approximately 10 to 20 days, where nitrogen was efficiently removed, or overland flow, which resulted in hydraulic residence times between 10 and 24 h with negligible nitrogen removal. Evapotranspiration concentrated solutes in the remaining subsurface-flow water, as evidenced by the increase in porewater chloride concentrations by up to a factor of 5 (Figure S2). Because evapotranspiration removed a large volume of water from the subsurface, porewater concentrations were normalized using conductivity measurements to allow for comparisons of mass removal, as described previously.<sup>12</sup> Swale cells illustrated the importance of subsurface flow:

35% of the applied nitrogen was removed in these cells because approximately 60% of the water never infiltrated the subsurface. As a result, these cells were excluded from our analyses. The full data set is available on Mendeley Data.<sup>37</sup>

**Nitrogen Cycling in the Horizontal Levee.** Throughout the 2 year monitoring period (June 2017–June 2019), nitrogen species were removed efficiently when hydraulic conditions were optimized.<sup>12</sup> Removal of nitrate, ammonium, and nitrite exceeded 98% when overland flow was eliminated. Only about 40% of the dissolved organic nitrogen (DON) was removed, although it only accounted for about 7% of the levee's influent nitrogen. Nitrate accounted for the majority of nitrogen lost in the levee, making up over 80% of influent nitrogen mass loading during the study (Figure 1a). Of the 104 kmol N removed, 77% was nitrate-N, with ammonium-N, nitrite-N, and DON accounting for the remaining 23%.

Porewater concentrations of nitrogen species (Figure 2) followed consistent trends with distance. In the first 5 m, removal of nitrate was rapid and significant (p < 0.03),<sup>12,17</sup> and ammonium and nitrite were removed within the first 2 m. Concentrations of DON decreased and then rebounded with no significant change (p = 0.13) within the first 15 m of the wetland, suggesting that initial removal of DON was followed by the release of DON from other processes (e.g., decomposition of plant residues). After 15 m, concentrations of all nitrogen species did not change significantly (p > 0.05). No significant differences (p > 0.05) were observed in porewater nitrogen concentrations seasonally or among wetland cell types,<sup>12</sup> except for nitrite, which exhibited slightly lower concentrations (p < 0.02) in wet meadow cells with coarse soils. The lower nitrite concentrations observed in cells with coarse topsoils may have been due to small, but significant, differences in pH. The median pH in the effluent was significantly (p < 0.005) lower, exiting cells with coarse topsoils (pH = 7.1) compared to fine topsoil cells (pH = 7.5). These observations are consistent with previous research.<sup>41</sup>

Mass balance calculations (Section S1 of the Supporting Information) indicated that of the 104 kmol of nitrogen removed during the monitoring period, approximately 81 kmol N (77%) was removed by conversion to gaseous forms (i.e.,  $N_{2(g)}$  and  $N_2O_{(g)}$ ) (Figure 1b). An additional 15 kmol N (14%) was assimilated by microorganisms or stored in particulate forms in the soil (section S1.1.3 of the Supporting Information). The remaining 8.8 kmol N (8%) was taken up into aboveground plant biomass (6.6 kmol N) or plant roots (2.2. kmol N).<sup>17</sup> Flows of nitrogen between different compartments were substantial (Figure 1b). Assuming that (1) soil nitrogen reached a steady state and (2) plant and litterfall turnover had reached maturity by the end of the monitoring period, these compartments (e.g., soil nitrogen and plant residues) do not represent important long-term sinks of wastewater-derived nitrogen. Based on the mass balances, only denitrification and anammox can explain the observed loss of dissolved nitrogen species.

In water-saturated subsurface wetlands where sediments are amended with organic substrates that receive high nitrate loadings, denitrification is often the dominant nitrate removal mechanism<sup>42-46</sup> because dissolved oxygen is rapidly consumed in the subsurface.<sup>47</sup> Moreover, even in systems where anammox and denitrification occur simultaneously, increasing organic matter correlates strongly with the relative importance of denitrification.<sup>45,48,49</sup> We therefore hypothesized that denitrification was the main nitrate removal pathway in the horizontal levee due to (1) an abundant supply of organic carbon (i.e., wood chips and decaying plants), (2) the rapid removal of nitrate with distance along the slope, (3) a significantly higher supply of nitrate relative to ammonium (i.e., the average influent concentrations were 1.8 and 0.21 mM respectively for nitrate and ammonium),<sup>12</sup> and (4) clear evidence of iron and sulfate reduction after nitrate was no longer detected. We also hypothesized that denitrification was fueled by a combination of electrons derived from various organic carbon sources and sulfide minerals, as discussed in subsequent sections.

In addition to mass balance calculations, we observed isotopic fractionation and microbiological evidence consistent with denitrification serving as the primary nitrate removal pathway. As described previously, fractionation of oxygen and nitrogen isotopes in nitrate in the horizontal levee followed Rayleigh kinetics and were strongly correlated,<sup>17</sup> which is typical when denitrification is the dominant nitrate removal process.<sup>50,51</sup> In contrast, when nitrogen removal by anammox is substantial, a decoupling of  $\delta^{18}$ O and  $\delta^{15}$ N in nitrate caused by reoxidation of nitrite during carbon fixation is expected.<sup>51</sup> Furthermore, we did not observe any anammox-specific genes (*hzs* and *hdh*) across the entire length of the wetland slope, diminishing the likelihood that anammox played a major role in nitrogen cycling (Figures S21 and S22).

Unlike anammox-specific genes, genes associated with full denitrification and DNRA metabolic pathways were present across the entire length of the slope at similar gene abundances (Figures S21 and S22). The similar abundances of genes specific to denitrification (*nirK*, *nirS*, *norBC*, and *nosZ*) and DNRA (*nirBD* and *nrfAH*) suggest that nitrogen was cycled through both metabolic pathways. However, of these processes, only denitrification can lead to the permanent nitrogen removal observed in the system because DNRA is only involved in internal cycling. Although we did not observe anammox genes or chemical indicators of anammox (e.g., nitrate production),<sup>30</sup> direct quantification methods (e.g., <sup>15</sup>N tracers)<sup>52</sup> would be needed to increase our confidence in the conclusion that denitrification accounted for all of the previously unexplained nitrate removal.

Ammonium accounted for 14% of the nitrogen mass removed. Its loss was likely explained primarily by microbial assimilation because microorganisms preferentially utilize ammonium over nitrate and DON.<sup>53</sup> Although anammox and feammox can also remove ammonium in anoxic wetland sediments,<sup>23,54,55</sup> these processes were not likely important for previously described reasons. Additionally, anammox relies on nitrite inputs and feammox forms nitrite as a product,<sup>54</sup> but we did not observe transient nitrite concentrations indicative of these processes (although nitrite rarely accumulates when sufficient labile organic carbon is present).<sup>10</sup> Based on the influent loading of ammonium, we estimated the maximum potential contribution of anammox and feammox to nitrogen removal at 27 and 14%, respectively. Additional experiments are needed to determine the importance of anammox and feammox more precisely.

Partial removal of DON was likely due to ammonification, followed by denitrification.<sup>10</sup> Despite residence times of more than 12 days in the subsurface,<sup>12</sup> only about 38% of DON was removed. This was consistent with past findings that indicated only about half of wastewater-derived DON is bioavailable to microorganisms in receiving waters.<sup>56</sup> Thus, we inferred that the fraction of DON originating from the wastewater that



Figure 3. Concentrations of  $Mn_{(aq)}$ ,  $Fe_{(aq)}$ , and sulfate in wet meadow cells with fine topsoils (a-c) and in wet meadow cells with coarse topsoils (d-f). Blue circles represent median concentrations from the winter and spring. Orange squares represent median concentrations in the summer and fall. Error bars represent 90th and 10th percentile values. Note the different vertial axis scales for each analyte.

passed through the horizontal levee consisted of forms that were resistant to microbial transformation. However, more labile forms of DON may have also been released within the horizontal levee from decomposition of plants,<sup>57</sup> leaching from soils,<sup>58</sup> and microbial biomass turnover.<sup>59</sup> Further characterization of the effluent DON is needed because the forms of DON produced in the horizontal levee (e.g., plant residues) could be more bioavailable in receiving waters than municipal wastewater-derived DON.

**Biogeochemical Cycles of Redox-Active Elements.** A complex web of interacting biogeochemical cycles influences nitrogen fate in horizontal levees because many of the microbial processes that drive these cycles compete for labile organic carbon. Our data suggest that organic carbon fueled a cascade of redox processes with progressively lower energy yields (i.e., reduction of  $O_2$  and  $NO_3^-$ , followed by Mn and Fe oxides, and then  $SO_4^{2-}$ ), as is expected in wetland sediments.<sup>60</sup> Although other electron donors may have also been produced from reactions of products of these processes (e.g., iron sulfides), the reducing power of the system was ultimately provided by the decomposition of organic matter in wood chips and wetland plants. These processes are described in greater detail in the following subsections.

Organic Carbon Cycling and Mass Balance. Five sources of organic carbon were relevant to the biogeochemistry of this system, including three solid forms: (1) wood chips mixed into the sediments during construction, (2) decaying plant biomass and root residues, and (3) soil-derived organic carbon, as well as two dissolved forms: (4) plant root exudates<sup>61</sup> and (5) wastewater-derived dissolved organic carbon. Prior to planting, the largest single reservoir of carbon was wood chips (approximately 7800 kmol C), accounting for approximately 66% of the organic carbon. An additional 4000 kmol C initially was present as soil-derived organic carbon. Before wastewater was introduced into the horizontal levee, organic carbon in the soil (i.e., from both wood chips and soil organic carbon) had decreased by approximately 15%. This decrease was likely due to a combination of oxidation of accessible forms of organic carbon by aerobic microorganisms<sup>62</sup> and leaching and export from organic carbon residues<sup>63</sup> during the period when the system was irrigated with sprinklers and the subsurface was unsaturated. See section S1.1.4 of the Supporting Information for further details.

Between 2017 and 2018, organic carbon from all sources increased by approximately 22% (p < 0.03; one-tail Mann-Whitney test) from around 7200 to 8200 kmol C. The increase in soil organic carbon suggested that organic carbon inputs from plants outpaced removal by oxidation in the watersaturated subsurface. Overall, approximately 1400 kmol C of plant biomass was produced over the 2 year study, with 18% stored belowground, as calculated from previously published biomass measurements.<sup>17</sup> These values were consistent with past studies in constructed wetlands,<sup>10</sup> which indicated that 200 to 7900 kmol C yr<sup>-1</sup> would be produced aboveground in a wetland of this size, with approximately 25% of total plant biomass stored belowground. Due to plant turnover (section S4 of the Supporting Information), nearly 90% of plant biomass produced during the 2 year study was either deposited as plant residues on the wetland surface or became decaying roots. Plant residues should be more readily assimilable than wood chips<sup>64</sup> due to their lower lignin content (e.g., <10% for plant residues<sup>65</sup> compared to >25% for wood chips).<sup>66</sup> Using measurements of carbon and nitrogen isotopes with depth (section S4 of the Supporting Information), we estimated that approximately 80% of the carbon in litter residues was integrated into the sediments over the monitoring period.



**Figure 4.** X-ray fluorescence spectromicroscopy data for samples collected from a coarse wet meadow cell in February 2020. Samples 1, 2, 3, and 4 were collected at distances of 1.5, 6, 23, and 38 m, respectively, from the inlet to the wetland, as demonstrated in (a). (b) The fraction of Fe-bearing minerals identified by LCF fitting of the Fe K-edge  $\mu$ XANES data for each sample broken down by Fe mineral groups and oxidation states. Sulfurbearing minerals are represented in blue, with iron carbonates in red, and all others in gray scale. (c–e) X-ray fluorescence elemental distribution maps of sulfur in samples 1 (8 × 8  $\mu$ m pixels), 2 (8 × 8  $\mu$ m pixels), and 3 (10 × 10  $\mu$ m pixels) that illustrate the change in the sulfur content with distance from the inlet. Scale bars are 500  $\mu$ m with the same gamma value used. Note that the detector was moved away from the sample for the 23 m sample (e).

Plant roots also likely exported significant amounts of organic carbon into the subsurface by releasing labile, low-molecular-weight compounds (i.e., exudates). Using root-mass-based relationships reported previously for plants used in constructed wetlands,<sup>61</sup> we estimated that 19 kmol C yr<sup>-1</sup> (95% CI = 6.3 kmol yr<sup>-1</sup>, 38 kmol C yr<sup>-1</sup>) were exuded by plants. Based on these estimates, exudates could supply approximately 16% of the electrons required for the heterotrophic processes in this system.

Wastewater-derived dissolved organic carbon (DOC) did not appear to be an important source of electrons for microbial processes. The mass of DOC did not change significantly (p =0.11) between the influent and effluent, consistent with past studies in wood chip-amended systems.<sup>15,67</sup> Organic carbon in wastewater effluent is typically recalcitrant: past measurements indicated that only 10–30% of wastewater effluent DOC is biodegradable.<sup>68,69</sup> For this reason, labile organic carbon is often added to stimulate denitrification in wastewater effluent.<sup>8,61</sup> We did not determine if wastewater DOC was consumed and replaced with organic carbon leached from soil organic carbon or plant residues, but even if we assume that 30% was consumed (29 kmol C),<sup>68,69</sup> it would account for less than 25% of the electrons required for heterotrophic processes.

Heterotrophic Processes and Mineral Formation. In the first 5 m of the wetland, nitrate (Figure 2) was consumed and there were modest increases in concentrations of  $Fe_{(aq)}$  and  $Mn_{(aq)}$  (Figure 3), likely due to reduction of Fe and Mn oxides within anaerobic biofilms after nitrate was depleted. Dissolved inorganic carbon increased (Figures S7 and S8), but modeling indicated that carbonate minerals remained undersaturated

(Figure S10). Sulfate reduction occurred as well, but sulfide minerals also remained undersaturated (Figure S11).

After nitrate was depleted, dissolved  $Fe_{(aq)}$  and  $Mn_{(aq)}$  concentrations increased, leveling out after 5 m in the coarse substrate cells. Sulfate removal indicated that sulfate reduction was likely the dominant heterotrophic process after nitrate was removed until sulfate was depleted between 5 and 20 m from the inlet. The distances at which we observed sulfate removal varied seasonally in the fine substrate cells (Figure 3) as well as among the different cell types (Figures 3 and S5), with the onset of sulfate removal occurring after 2 m in the fine substrate cells during the winter.

Assuming that sulfate was converted to sulfide, the cells were likely supersaturated with respect to sulfide minerals after around 2–7 m, depending upon the cell type (Figure S11). Research conducted in other organic carbon-amended subsurface flow wetlands indicates that sulfate is typically reduced to sulfide, resulting in the formation of acid-volatile sulfides.<sup>70</sup> Additionally, iron was abundant in the wetland sediments, constituting approximately 2.5% of the sediment by weight (Section S1 of the Supporting Information), with structural iron, iron silicates, and iron oxides abundant (Figure 4b) based on Fe XANES measurements (Figure S18; Table S9). For these reasons, we hypothesized that iron and sulfate reduction resulted in the significant formation of Fe(II) sulfide minerals (up to approximately 19 kmol).

Changes in aqueous measurements of those species were consistent with black coatings observed on sediments collected from depths below 75 cm at distances at 6 and 23 m from the inlet (Figure S12)<sup>71,72</sup> as well as with mineral characterization in the gravel sublayer during the winter using XANES (Figure



**Figure 5.** Concentrations ([C]) of (a) nitrate (blue circles) and sulfate (orange squares) and (b)  $Fe_{(aq)}$  (gray triangles), ammonium (green circles), and nitrite (black squares) in porewater samples collected from the gravel and sand layers of the subsurface in a fine wet meadow cell on April 10, 2019. Note the difference in vertical axes scales. Data points represent individual samples, and lines represent moving averages.

4). Sulfide minerals such as greigite  $(Fe(II)Fe(III)_2S_{3(s)})$ , the most frequently identified sulfide mineral, as well as pyrite  $(FeS_{2(s)})$  and arsenopyrite  $(FeAsS_{(s)})$ , were identified in February 2020 at distances of 6, 23, and 38 m along the slope (Figure 4b; Table S9). Elemental mapping indicated that the sulfur content in the gravel sublayer increased substantially between 1.5 and 6 m (Figure 4b,c). At 23 m, the sulfur content saturated the detector and sulfur mapping had to be conducted with the detector at a greater distance from the sample (Figure 4e). These findings suggest that the majority of sulfur precipitated onto sediments beyond 1.5 m from the inlet. Based on rapid sulfate removal observed during the summer, we suspect that small amounts of sulfide minerals may also have formed on sediments within the first few meters (Figure S13).

After sulfate was depleted, fermentation and methanogenesis, along with continued Fe(III) reduction, were likely the most important biogeochemical processes. This hypothesis is consistent with the observed ubiquity of fermenters and methanogens in soils and sediments globally<sup>73</sup> and the highly reducing and anoxic conditions observed in the second half of the horizontal levee slope, which favor their activity.<sup>73</sup> Between 25 and 45 m, concentrations of dissolved inorganic carbon nearly doubled (Figure S8) primarily due to removal of water by evapotranspiration (Figure S2). Under these conditions, stable porewater  $Fe_{(aq)}$  and  $Mn_{(aq)}$  concentrations were consistent with the presence of carbonate minerals. Coalingite, a Mg/Fe(III) carbonate, was identified using Fe XANES at distances of 23 and 38 m (Figure 4b and Table S9). Despite the abundant presence of Fe(III) and Mn(IV) minerals in the subsurface, water loss by evapotranspiration, and production of inorganic carbon by microbial processes, dissolved Mn concentrations in porewater only increased by 23% (p < 0.01; one-tailed *t*-test) in this part of the slope and dissolved Fe concentrations did not change (p = 0.17; two-tailed Mann-Whitney test) (Figure S5), suggesting that  $Mn_{(aq)}$  and  $Fe_{(aq)}$ were precipitating as Mn(II) and Fe(II) minerals.

At the start of the experiment, approximately 39 kmol Mn and 2300 kmol Fe were present in the wetland sediments. Based on porewater concentrations of  $Mn_{(aq)}$  and  $Fe_{(aq)}$  collected at the outlet from the wetland, we estimate that only about 9 and 0.3% of these metals were exported from the wetland, respectively, during the study. Porewater Mn and Fe concentrations did not vary seasonally (p > 0.05; Wilcoxon signed-rank test) but were significantly lower in cells planted with willows (Figure S5; Section S2 of the Supporting Information). Based on these export rates, we estimate that

Mn and Fe oxides would be exhausted after approximately 20 and 750 years, respectively.

Autotrophic Sulfide-Driven Denitrification and DNRA. In addition to the evidence of heterotrophic processes discussed above, we frequently observed increasing sulfate concentrations in the first 5 m of the slope coincident with nitrate removal, particularly during winter and spring (Figure 3c,f, blue circles). This trend was more evident in data collected from the fine substrate wetland cells. For example, in April 2019, nitrate concentrations dropped from 2.1 mM to less than 0.01 mM in the first 5 m of a fine wet meadow cell over the same distance that sulfate concentrations increased from approximately 0.45 to 1.0 mM (Figure 5). The inverse correlation ( $r^2 = 0.97$ ; p = 0.002) between sulfate and nitrate in these observations suggests that autotrophic sulfide-driven denitrification and/or DNRA occurred.<sup>25,74</sup> These processes are fueled by sulfide minerals (e.g., acid-volatile sulfides), which, as shown previously, were formed during iron and sulfate reduction (Section S3.2 of the Supporting Information). These minerals likely formed in the first 5 m of the subsurface during summer and fall when biological activity was the highest and subsequently were consumed by sulfide-driven denitrification and/or DNRA in the winter and spring. The likelihood of sulfide-driven denitrification and/or DNRA processes is supported by the ubiquity of dissimilatory sulfur reduction and oxidation genes along the length of the wetland, in conjunction with denitrification and DNRA genes (Figures S22 and S23). Differences in flow rates or microbial biomass between substrate types may have caused this phenomenon to be less pronounced in the coarse substrate cells. The apparent differences between cell types may have also been due to the lower sampling resolution in the coarse substrate cells in the winter.

Sulfide-driven DNRA can link nitrate reduction and anammox in constructed wetlands,<sup>25</sup> but ammonium concentrations in our system did not increase as nitrate was removed (Figure 5b), as would have been expected if DNRA was important. Rather, our results were more likely attributable to sulfide-driven denitrification—a process that has been observed in marine<sup>75</sup> and riverine sediments<sup>76</sup> and in constructed wetlands with high sulfate loading.<sup>70,77</sup> Many sulfide-oxidizing denitrifiers can also oxidize Fe(II), which may explain the low  $Fe_{(aq)}$  concentrations observed in these samples.<sup>78</sup>

In the horizontal levee, these trends were seasonal, with release of sulfate in the first few meters only observed to a significant degree in winter and spring. Due to the seasonality of these trends (Figure 3), we hypothesized that autotrophic sulfide-driven denitrification was more important in winter. Sulfate reduction occurred in the first 3 m of the subsurface during summer, particularly in fine substrate cells (Figure 3c), likely leading to the formation of Fe(II) sulfides that fueled autotrophic denitrification during winter and spring (Figure S13). Oxidation of sulfide by denitrifiers during winter may partly explain the low sulfur content (Figure 4c) and the lack of sulfide minerals identified at 1.5 m along the slope (Figure 4b, Table S9).

The distance at which nitrate removal occurred was not significantly different seasonally (p = 0.78; Figure S6; Section S2.3 of the Supporting Information). This contrasts with previously published research in constructed wetlands, in which nitrate removal rates slowed as the temperature decreased in surface-flow<sup>18</sup> and subsurface wetlands.<sup>10</sup> On the basis of water temperature changes between summer  $(21 \pm$ 0.8 °C) and winter  $(17 \pm 1.3 \text{ °C})$ , we would have expected nearly a 40% decrease in nitrate removal rates, which would have extended the length of the denitrifying zone of the wetland from approximately 4 to 7 m. However, the lack of seasonality observed in nitrate removal may have been because an increase in sulfide-driven denitrification, a process that typically exhibits a weaker temperature dependence,<sup>19,75</sup> compensated for more temperature-sensitive removal processes.

The ability of the horizontal levee to remove dissolved nitrogen efficiently during different seasons suggests that horizontal levees may not exhibit the dramatic diminution in performance that has previously led some decision-makers to discount the use of nature-based solutions. Horizontal levees may also be attractive for nitrogen removal when wastewater contains high sulfate concentrations (e.g., reverse osmosis concentrate). Moreover, the amendment of horizontal levee sediments with Fe(III) oxides or FeS<sub>(s)</sub> could stimulate sulfide-driven nitrate removal in constructed wetlands, as has been reported previously,<sup>79,80</sup> and may help prevent seasonal performance fluctuations.

Based on changes in sulfate and nitrate concentrations and the stoichiometry of sulfide-driven denitrification, we estimated that 130 Keq of the approximately 440 Keq of electron donors required for nitrate reduction during winter were supplied by the oxidation of sulfide minerals.<sup>74,81</sup> This suggests that nearly one-third of wintertime denitrification and 18% of overall nitrate removal during winter were fueled by Fe(II) sulfides. Over the entire year, this would account for approximately 10% of nitrate removal. Additionally, although sulfide deposits may become less reactive as they are converted to more stable forms (e.g., pyrite),<sup>82–84</sup> there is growing evidence that they still can provide electrons for denitrification.<sup>80,85</sup>

**Implications for the Horizontal Levee Design.** To illustrate how biogeochemistry can inform horizontal levee design, we used our data to evaluate the role of different electron donors and acceptors in nitrogen removal (Section S1 of the Supporting Information). Approximately 1000 Keq of electrons was transferred to the most abundant electron acceptors (i.e.,  $O_2$ ,  $NO_3^-$ ,  $NO_2^-$ , Mn(IV), Fe(III),  $SO_4^{2-}$ ) during the 2 year study. Known electron donors provided sufficient reductants to drive these processes (Figure S4). Among the major electron acceptors, we estimated that approximately 670 Keq (about 50%) of the electrons supplied by electron donors was used to convert  $NO_3^-$  and  $NO_2^-$  into  $N_{2(g)}$  and trace amounts of  $N_2O_{(g)}$ . Metal sulfides provided

approximately 10% of the reductants needed for this process, primarily during colder seasons when rates of heterotrophic denitrification decreased. Approximately 20% of organic carbon consumption (54 kmol C) was not accounted for by the monitored electron acceptors (Figure S4) and may have been due to methanogenesis or fermentation (Section S1.2 of the Supporting Information).<sup>86</sup>

If all of the added carbon in the wood chips were bioavailable, it could supply 44,000 Keq of reducing power, enough to denitrify all of the nitrate applied to the experimental system for approximately 65 years. However, complete mineralization is unlikely because the high lignin content of wood chips<sup>87</sup> assures that not all of the organic carbon can be released quickly.<sup>88</sup> The results from previous studies of wood chip-amended treatment systems indicate that biological activity stimulated by wood chips decreases over decadal timescales,<sup>67</sup> likely as more easily extracted fractions of wood chip carbon are exhausted. Thus, we expect organic carbon release from wood chips to decline over time with some fraction of the wood persisting for over a century. Considering the amount of organic carbon in the soil available at the beginning of the experiment (~8200 kmol C; including wood chips, soil organic matter, and decaying root biomass) and the rate of organic carbon consumption ( $\sim$ 120 kmol yr<sup>-1</sup>; Section S1.1.4 of the Supporting Information), we estimate that accessible organic carbon in the soil would be depleted in approximately 50 years without additional inputs. However, plants growing on the surface of the wetland produce litter (approximately 1400 kmol C  $yr^{-1}$ ) that could replenish consumed organic carbon.<sup>17</sup> Additionally, reducing power from plant root exudates<sup>61</sup> and iron sulfide deposits may also fuel microbial processes like denitrification.

To assess the ability of a horizontal levee to remove nitrate in the long term, we developed a model that balances production of total electron donors in the horizontal levee against their consumption through metabolic transfers of electrons to electron acceptors (Section S1.3 of the Supporting Information; Table S4). We estimate that plants would produce around 1000 kmol C yr<sup>-1</sup> after they reach maturity based on observed biomass production rates reported in our previous study.<sup>17</sup> If just 10% of this annual biomass production is ultimately available to microbes in the subsurface, the ability of the system to completely remove nitrogen would be sustained without the need to amend the system with electron donors (e.g., wood chips).

The size of alternative electron acceptor reservoirs can also impact the amount of organic carbon available for nitrate removal by denitrification. Reactive Mn(III)/Mn(IV) and Fe(III) oxides in construction materials could serve as sinks for organic carbon that would otherwise be used for denitrification, similar to Mn and Fe oxides in marine sediments.<sup>89</sup> High sulfate reduction rates could also deplete the reservoir of organic carbon if sulfate-rich wastewater streams (i.e., reverse osmosis concentrate from wastewater reuse projects) were applied to horizontal levees. However, iron and sulfur may also be beneficial if they are stoichiometrically balanced to avoid free sulfide-induced phytotoxicity<sup>90</sup> or export of reduced forms of those elements from the system. Not only would large inputs of Fe(III) and sulfate favor Fe(II) sulfide formation in these systems, creating a reservoir of electrons that can sustain high rates of nitrogen removal in the winter, but also Fe(III) and sulfate can inhibit the formation of methane<sup>91-93</sup> and

methylmercury,  $^{94,95}$  which are often concerns in constructed wetlands.

Despite the complex biogeochemistry of these systems, our results indicate that nitrogen removal is primarily achieved through microbial conversion to gaseous forms via denitrification. The dominance of denitrification that we observed is consistent with past studies of nitrogen removal from wastewater effluent in subsurface-constructed wetlands.<sup>10,44,96</sup> For example, in subsurface wetland microcosms amended with plant litter and fed with high concentrations of nitrate, 54-91% of nitrogen removal was attributed to denitrification.<sup>44</sup> Our results were also consistent with research on nitrogen removal in similar nature-based treatment systems receiving nitrate-polluted water (e.g., denitrification walls<sup>14,15</sup> and managed riparian wetlands).<sup>13</sup> We observed that sulfide minerals are an important source of electrons, particularly in winter when sulfide-driven denitrification prevented the seasonal fluctuations in nitrate removal that have been reported in constructed wetlands.<sup>10,18,96</sup> This is consistent with studies indicating that sulfide-driven denitrification improves nitrate removal in groundwater<sup>19,78,85</sup> and subsurface wetlands.<sup>79,80</sup> Last, the model we developed suggests that nitrogen removal may be sustained by the suite of electron sources in the subsurface. Nitrate removal can be optimized when design and operational decisions appropriately consider the complex biogeochemistry of horizontal levees.

# ASSOCIATED CONTENT

# **Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c07512.

Methods and results for mass and electron balances; additional details on nitrogen and elemental cycling; visual MINTEQ modeling; methods for modeling production, decomposition, and reintegration of plant residues into wetland sediments; elemental distribution maps, iron valence scatter plots, least-squares linear combination fittings of Fe  $\mu$ XANES data, and other details on XANES analysis; and methods for determining microbial gene abundances and microbial gene abundance data (PDF)

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#### **Author Contributions**

The manuscript was written through contributions as delineated below. All authors have given approval to the final version of the manuscript. A.C.: conceptualization, methodology, validation, investigation, formal analysis, data curation, writing—original draft, and visualization; A.S.: methodology, formal analysis, investigation, writing—review and editing, and visualization; E.G.: methodology, formal analysis, investigation, writing—review and editing, and visualization; S.B.: methodology and writing—review and editing; S.F.: methodology, formal analysis, investigation, data curation, visualization, and writing—review and editing; L.A.-C.: resources, writing review and editing, supervision, project administration, and funding acquisition; D.S.: conceptualization, resources, writing—review and editing, supervision, project administration, and funding acquisition.

#### Notes

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# REFERENCES

(1) 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. Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science* **2008**, *320*, 889–892.

(2) Seitzinger, S.; Harrison, J. A.; Böhlke, J. K.; Bouwman, A. F.; Lowrance, R.; Peterson, B.; Tobias, C.; Drecht, G. V. Denitrification across landscapes and waterscapes: a synthesis. *Ecol. Appl.* **2006**, *16*, 2064–2090.

(3) Gruber, N.; Galloway, J. N. An earth-system perspective of the global nitrogen cycle. *Nature* **2008**, *451*, 293–296.

(4) Anderson, D. M.; Glibert, P. M.; Burkholder, J. M. Harmful algal blooms and eutrophication: nutrient sources, composition and consequences. *Estuaries* **2002**, *25*, 704–726.

(5) Xiao, X.; Agustí, S.; Pan, Y.; Yu, Y.; Li, K.; Wu, J.; Duarte, C. M. Warming amplifies the frequency of harmful algal blooms with eutrophication in Chinese coastal waters. *Environ. Sci. Technol.* **2019**, *53*, 13031–13041.

(6) Heiskanen, A.-S.; Bonsdorff, E.; Joas, M. Chapter 20—Baltic Sea: A Recovering Future from Decades of Eutrophication. *Coasts and Estuaries: The Future*; Elsevier, 2019; pp 343–362.

(7) Ko, J.-Y.; Day, J. W.; Lane, R. R.; Day, J. N. A comparative evaluation of money-based and energy-based cost-benefit analyses of tertiary municipal wastewater treatment using forested wetlands vs. sand filtration in Louisiana. *Ecol. Econ.* **2004**, *49*, 331–347.

(8) Tchobanoglous, G.; Burtono, F. L.; Stensel, H. D. *Wastewater Engineering: Treatment and Reuse*, 5th ed.; McGraw-Hill: New York City, NY, 2013.

(9) Seifollahi-Aghmiuni, S.; Nockrach, M.; Kalantari, Z. The potential of wetlands in achieving the sustainable development goals of the 2030 agenda. *Water* **2019**, *11*, 609.

(10) Kadlec, R. H., Wallace, S. D. *Treatment Wetlands*. 2nd ed.; CRC Press: Boca Raton, FL, 2009.

(11) Morris, J. T.; Shaffer, G. P.; Nyman, J. A. Brinson review: perspectives on the influence of nutrients on the sustainability of coastal wetlands. *Wetlands* **2013**, *33*, 975–988.

(12) Cecchetti, A. R.; Stiegler, A. N.; Graham, K. E.; Sedlak, D. L. The horizontal levee: a multi-benefit nature-based treatment system that improves water quality while protecting coastal levees from the effects of sea level rise. *Water Res.: X* 2020, *7*, 100052.

(13) Hill, A. R. Stream Chemistry and Riparian Zones. In *Streams and Ground Waters*; Jones, J. B., Mulholland, P. J., Eds.; Academic Press: London, 2000.

(14) Schipper, L. A.; Barkle, G. F.; Hadfield, J. C.; Vojvodic-Vukovic, M.; Burgess, C. P. Hydraulic constraints on the performance of a groundwater denitrification wall for nitrate removal from shallow groundwater. *J. Contam. Hydrol.* **2004**, *69*, 263–279.

(15) Schmidt, C. A.; Clark, M. W. Efficacy of a denitrification wall to treat continuously high nitrate loads. *Ecol. Eng.* **2012**, *42*, 203–211.

(16) Harris-Lovett, S.; Lienert, J.; Sedlak, D. A mixed-methods approach to strategic planning for multi-benefit regional water infrastructure. *J. Environ. Manage.* **2019**, 233, 218–237.

(17) Cecchetti, A. R.; Sytsma, A.; Stiegler, A. N.; Dawson, T. E.; Sedlak, D. L. Use of stable nitrogen isotopes to track plant uptake of nitrogen in a nature-based treatment system. *Water Res.: X* **2020**, *9*, 100070.

(18) Jasper, J. T.; Jones, Z. L.; Sharp, J. O.; Sedlak, D. L. Nitrate removal in shallow, open-water treatment wetlands. *Environ. Sci. Technol.* 2014, 48, 11512–11520.

(19) Jørgensen, C.J.; Jacobsen, O.S.; Elberling, B.; Aamand, J. Microbial oxidation of pyrite coupled to nitrate reduction in anoxic groundwater sediment. *Environ. Sci. Technol.* **2009**, *43*, 4851–4857.

(20) Guo, W.; Wen, Y.; Chen, Y.; Zhou, Q. Sulfur cycle as an electron mediator between carbon and nitrate in constructed wetland microcosm. *Front. Environ. Sci. Eng.* **2020**, *14*, 57.

(21) Liu, W.; Rahaman, M. H.; Mąkinia, J.; Zhai, J. Coupling transformation of carbon, nitrogen and sulfur in a long-term operated full-scale constructed wetland. *Sci. Total Environ.* **2021**, 777, 146016.

(22) Burgin, A. J.; Hamilton, S. K. Have we overemphasized the role of denitrification in aquatic ecosystems? a review of nitrate removal pathways. *Front. Ecol. Environ.* **2007**, *5*, 89–96.

(23) Yang, W. H.; Weber, K. A.; Silver, W. L. Nitrogen loss from soil through anaerobic ammonium oxidation coupled to iron reduction. *Nat. Geosci.* **2012**, *5*, 538–541.

(24) Giblin, A.; Tobias, C.; Song, B.; Weston, N.; Banta, G.; Rivera-Monroy, V. The importance of dissimilatory nitrate reduction to ammonium (DNRA) in the nitrogen cycle of coastal ecosystems. *Oceanography* **2013**, *26*, 124–131.

(25) Jones, Z.L.; Jasper, J.T.; Sedlak, D.L.; Sharp, J.O. Sulfideinduced dissimilatory nitrate reduction to ammonium supports anaerobic ammonium oxidation (Anammox) in an open-water unit process wetland. *Appl. Environ. Microbiol.* **201**7, *83*, No. e00782.

(26) Wang, Q.; Rogers, M. J.; Ng, S. S.; He, J. Fixed nitrogen removal mechanisms associated with sulfur cycling in tropical wetlands. *Water Res.* **2021**, *189*, 116619.

(27) Groffman, P. M.; Altabet, M. A.; Böhlke, J. K.; Butterbach-Bahl, K.; David, M. B.; Firestone, M. K.; Giblin, A. E.; Kana, T. M.; Nielsen, L. P.; Voytek, M. A. Methods for measuring denitrification: diverse

approaches to a difficult problem. *Ecol. Appl.* **2006**, *16*, 2091–2122. (28) Reinhardt, M.; Müller, B.; Gächter, R.; Wehrli, B. Nitrogen removal in a small constructed wetland: an isotope mass balance approach. *Environ. Sci. Technol.* **2006**, *40*, 3313–3319.

(29) Steidl, J.; Kalettka, T.; Bauwe, A. Nitrogen retention efficiency of a surface-flow constructed wetland receiving tile drainage water: a case study from north-eastern Germany. *Agric., Ecosyst. Environ.* **2019**, 283, 106577.

(30) Rampuria, A.; Gupta, A. B.; Brighu, U. Nitrogen transformation processes and mass balance in deep constructed wetlands treating sewage, exploring the anammox contribution. *Bioresour. Technol.* **2020**, 314, 123737.

(31) Alongi, D. M. Nitrogen cycling and mass balance in the world's mangrove forests. *Nitrogen* **2020**, *1*, 167–189.

(32) Almaraz, M.; Wong, M. Y.; Yang, W. H. Looking back to look ahead: a vision for soil denitrification research. *Ecol* **2020**, *101*, No. e02917.

(33) Thomas, D. H.; Rey, M.; Jackson, P. E. Determination of inorganic cations and ammonium in environmental waters by ion chromatography with a high-capacity cation-exchange column. *J. Chromatogr. A* **2002**, *956*, 181–186.

(34) American Public Health Association (APHA). Standard Methods for the Examination of Water and Wastewater, 22nd ed.; American Public Health Association, American Water Works Association, Water Environment Foundation: Washington, DC, 2012. (35) Sigman, D. M.; Casciotti, K. L.; Andreani, M.; Barford, C.; Galanter, M.; Böhlke, J. K. A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. Anal. Chem. 2001, 73, 4145–4153.

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

(37) Cecchetti, A.; Stiegler, A.; Sytsma, A.; Bandaru, S.; Gonthier, E.; Graham, K.; Boehm, A. B.; Fakra, S.; Dawson, T.; Sedlak, D. Monitoring Data from a Pilot-Scale Horizontal Levee. *Mendeley Data*, 2021, version 3.

(39) Marcus, M. A.; MacDowell, A. A.; Celestre, R.; Manceau, A.; Miller, T.; Padmore, H. A.; Sublett, R. E. Beamline 10.3.2 at ALS: a hard X-ray microprobe for environmental and materials sciences. *J. Synchrotron Radiat.* **2004**, *11*, 239–247.

(40) Zaiontz, C. Real Statistics Using Excel. 2018, Accessed from: www.real-statistics.com.

(41) Lim, N. Y. N.; Frostegård, Å.; Bakken, L. R. Nitrite kinetics during anoxia: the role of abiotic reactions versus microbial reduction. *Soil Biol. Biochem.* **2018**, *119*, 203–209.

(42) Ingersoll, T. L.; Baker, L. A. Nitrate removal in wetland microcosms. *Water Res.* **1998**, *32*, 677–684.

(43) Wen, Y.; Chen, Y.; Zheng, N.; Yang, D.; Zhou, Q. Effects of plant biomass on nitrate removal and transformation of carbon sources in subsurface-flow constructed wetlands. *Bioresour. Technol.* **2010**, *101*(), DOI: 10.1016/j.biortech.2010.04.068.

(44) Chen, Y.; Wen, Y.; Zhou, Q.; Vymazal, J. Effects of plant biomass on nitrogen transformation in subsurface-batch constructed wetlands: a stable isotope and mass balance assessment. *Water Res.* **2014**, *63*, 158–167.

(45) Rambags, F.; Tanner, C. C.; Schipper, L. A. Denitrification and anammox remove nitrogen in denitrifying bioreactors. *Ecol. Eng.* **2019**, *138*, 38–45.

(46) Saeed, T.; Sun, G. A review on nitrogen and organics removal in subsurface flow constructed wetlands: dependency on environmental parameters, operating conditions and supporting media. *J. Environ. Manage.* **2012**, *112*, 429–448.

(47) Rivett, M. O.; Buss, S. R.; Morgan, P.; Smith, J. W. N.; Bemment, C. D. Nitrate attenuation in groundwater: a review of biogeochemical controlling processes. *Water Res.* **2008**, *42*, 4215–4232.

(48) Li, H.; Tao, W. Efficient ammonia removal in recirculating vertical flow constructed wetlands: complementary roles of anammox and denitrification in simultaneous nitritation, anammox and denitrification process. *Chem. Eng. J.* **2017**, *317*, 972–979.

(49) Chen, D.; Gu, X.; Zhu, W.; He, S.; Wu, F.; Huang, J.; Zhou, W. Denitrification- and anammox-dominant simultaneous nitrification, anammox and denitrification (SNAD) process in subsurface flow constructed wetlands. *Bioresour. Technol.* **2019**, *271*, 298–305.

(50) Kendall, C. Chapter 16—Tracing Nitrogen Sources and Cycling in Catchments. In *Isotope Tracers in Catchment Hydrology*; Elsevier, 1998; pp 519–576.

(51) Dähnke, K.; Thamdrup, B. Isotope fractionation and isotope decoupling during anammox and denitrification in marine sediments. *Limnol. Oceanogr.* **2016**, *61*, 610–624.

(52) Erler, D. V.; Eyre, B. D.; Davison, L. The contribution of anammox and denitrification to sediment  $N_2$  production in a surface flow constructed wetland. *Environ. Sci. Technol.* **2008**, *42*, 9144–9150.

(53) Inamori, R.; Wang, Y.; Yamamoto, T.; Zhang, J.; Kong, H.; Xu, K.; Inamori, Y. Seasonal effect on  $N_2O$  formation in nitrification in constructed wetlands. *Chemosphere* **2008**, 73, 1071–1077.

(54) Li, X.; Hou, L.; Liu, M.; Zheng, Y.; Yin, G.; Lin, X.; Cheng, L.; Li, Y.; Hu, X. Evidence of nitrogen loss from anaerobic ammonium oxidation coupled with ferric iron reduction in an intertidal wetland. *Environ. Sci. Technol.* **2015**, *49*, 11560–11568.

(55) Shuai, W.; Jaffé, P. R. Anaerobic ammonium oxidation coupled to iron reduction in constructed wetland mesocosms. *Sci. Total Environ.* **2019**, *648*, 984–992.

(56) Pehlivanoglu, E.; Sedlak, D. L. Bioavailability of wastewaterderived organic nitrogen to the alga Selenastrum Capricornutum. *Wat. Res.* **2004**, *38*, 3189–3196.

(57) van Kessel, C.; Clough, T.; van Groenigen, J. W. Dissolved organic nitrogen: an overlooked pathway of nitrogen loss from agricultural systems? *J. Environ. Qual.* **2009**, *38*, 393–401.

(58) Li, L.; Davis, A. P. Urban stormwater runoff nitrogen composition and fate in bioretention systems. *Environ. Sci. Technol.* **2014**, *48*, 3403–3410.

(59) Westerhoff, P.; Mash, H. Dissolved organic nitrogen in drinking water supplies: a review. J. Water Supply: Res. Technol.-AQUA 2002, 51, 415–448.

(60) Rysgaard, S.; Fossing, H.; Jensen, M. M. Organic matter degradation through oxygen respiration, denitrification, and manganese, iron and sulfate reduction in marine sediments (the Kattegat and the Skagerrak). *Ophelia* **2001**, *55*, 77–91.

(61) Zhai, X.; Piwpuan, N.; Arias, C. A.; Headley, T.; Brix, H. Can root exudates from emergent wetland plants fuel denitrification in subsurface flow constructed wetland systems? *Ecol. Eng.* **2013**, *61*, 555–563.

(62) McLatchey, G. P.; Reddy, K. R. Regulation of organic matter decomposition and nutrient release in a wetland soil. *J. Environ. Qual.* **1998**, *27*, 1268–1274.

(63) Abusallout, I.; Hua, G. Characterization of dissolved organic carbon leached from a woodchip bioreactor. *Chemosphere* **2017**, *183*, 36–43.

(64) Stelzer, R. S.; Thad Scott, J.; Bartsch, L. A.; Parr, T. B. Particulate organic matter quality influence nitrate retention and denitrification in stream sediments: evidence from a carbon burial experiment. *Biogeochem* **2014**, *119*, 387–402.

(65) Moran, M. A.; Hodson, R. E. Bacterial secondary production on vascular plant detritus: relationships to detritus composition and degradation rate. *Appl. Environ. Microbiol.* **1989**, *55*, 2178–2189.

(66) Gašparovič, L.; Koreňová, Z.; Jelemenský, L. Kinetic study of wood chips decomposition by TGA. *Chem. Pap.* **2010**, *64*, 174–181.

(67) Schipper, L. A.; Cameron, S. C.; Warneke, S. Nitrate removal from three different effluents using large-scale denitrification beds. *Ecol. Eng.* **2010**, *36*, 1552–1557.

(68) Lai, T. M.; Shin, J.-K.; Hur, J. Estimating the biodegradability of treated sewage samples using synchronous fluorescence spectra. *Sensors* **2011**, *11*, 7382–7394.

(69) Wang, M.; Chen, Y. Generation and characterization of DOM in wastewater treatment processes. *Chemosphere* **2018**, 201, 96–109.

(70) Chen, Y.; Wen, Y.; Zhou, Q.; Huang, J.; Vymazal, J.; Kuschk, P. Sulfate removal and sulfur transformation in constructed wetlands: the role of filling material and plant biomass. *Water Res.* **2016**, *102*, 572–581.

(71) Cornwell, J. C.; Morse, J. W. The characterization of iron sulfide minerals in anoxic marine sediments. *Mar. Chem.* **1987**, *22*, 193–206.

(72) Wang, F.; Chapman, P. M. Biological implications of sulfide in sediment-a review focusing on sediment toxicity. *Environ. Toxicol. Chem.* **1999**, *18*, 2526–2532.

(73) Angel, R.; Claus, P.; Conrad, R. Methanogenic archaea are globally ubiquitous in aerated soils and become active under wet anoxic conditions. *ISME J.* **2012**, *6*, 847–862.

(74) Zhang, Y.-C.; Slomp, C. P.; Broers, H. P.; Bostick, B.; Passier, H. F.; Böttcher, M. E.; Omoregie, E. O.; Lloyd, J. R.; Polya, D. A.; van Cappellen, P. Isotopic and microbiological signatures of pyrite-driven denitrification in a sandy aquifer. *Chem. Geol.* **2012**, 300–301, 123–132.

(75) Zhang, M.; Zhang, T.; Shao, M. F.; Fang, H. H. P. Autotrophic denitrification in nitrate-induced marine sediment remediation and Sulfurimonas denitrificans-like bacteria. *Chemosphere* **2009**, *76*, 677–682.

(76) Yang, X.; Huang, S.; Wu, Q.; Zhang, R. Nitrate reduction coupled with microbial oxidation of sulfide in river sediment. *J. Soils Sediments* **2012**, *12*, 1435–1444.

(77) Guo, W.; Cecchetti, A. R.; Wen, Y.; Zhou, Q.; Sedlak, D. L. Sulfur cycle in a wetland microcosm: extended <sup>34</sup>S-stable isotope analysis and mass balance. *Environ. Sci. Technol.* **2020**, *54*, 5498–5508.

(78) Torrentó, C.; Cama, J.; Urmeneta, J.; Otero, N.; Soler, A. Denitrification of groundwater with pyrite and Thiobacillus denitrificans. *Chem. Geol.* **2010**, *278*, 80–91.

(79) Si, Z.; Song, X.; Wang, Y.; Cao, X.; Wang, Y.; Zhao, Y.; Ge, X. Natural pyrite improves nitrate removal in constructed wetlands and makes wetland a sink for phosphorus in cold climates. J. Cleaner Prod. 2021, 280, 124304.

(80) Xu, Z.; Qiao, W.; Song, X.; Wang, Y. Pathways regulating the enhanced nitrogen removal in a pyrite based vertical-flow constructed wetland. *Bioresour. Technol.* **2021**, *325*, 124705.

(81) Cardoso, R. B.; Sierra-Alvarez, R.; Rowlette, P.; Flores, E. R.; Gómez, J.; Field, J. A. Sulfide oxidation under chemolithoautotrophic denitrifying conditions. *Biotechnol. Bioeng.* **2006**, *95*, 1148–1157.

(82) Berner, R. A. Thermodynamic stabilities of sedimentary iron sulfides. *Am. J. Sci.* **1967**, *265*, 773–785.

(83) Canfield, D. E. Reactive iron in marine sediments. *Geochim. Cosmochim. Acta* 1989, 53, 619–632.

(84) Furukawa, Y.; Barnes, H. L. Chapter 10: Reactions forming pyrite from precipitated amorphous ferrous sulfide. *Geochemical Transformations of Sedimentary Sulfur*; ACS Symposium Series, 1995; Vol. 612, pp 194–205.

(85) Torrentó, C.; Urmeneta, J.; Otero, N.; Soler, A.; Viñas, M.; Cama, J. Enhanced denitrification in groundwater and sediments from a nitrate-contaminated aquifer after addition of pyrite. *Chem. Geol.* **2011**, 287, 90–101.

(86) Conrad, R. Soil microorganisms as controllers of atmospheric trace gases (H<sub>2</sub>, CO, CH<sub>4</sub>, OCS, N<sub>2</sub>O, and NO). *Microbiol. Rev.* **1996**, 60, 609–640. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC239458/pdf/600609.pdf

(87) Hang, Q.; Wang, H.; Chu, Z.; Ye, B.; Li, C.; Hou, Z. Application of plant carbon source for denitrification by constructed wetland and bioreactor: review of recent development. *Environ. Sci. Pollut. Res.* **2016**, *23*, 8260–8274.

(88) García, J.; Rousseau, D. P. L.; Morató, J.; Lesage, E.; Matamoros, V.; Bayona, J. M. Contaminant removal processes in subsurface-flow constructed wetlands: a review. *Crit. Rev. Environ. Sci. Technol.* **2010**, *40*, 561–661.

(89) Canfield, D. E.; Thamdrup, B.; Hansen, J. W. The anaerobic degradation of organic matter in Danish coastal sediments: iron reduction, manganese reduction, and sulfate reduction. *Geochim. Cosmochim. Acta* **1993**, *57*, 3867–3883.

(90) Lamers, L. P. M.; Govers, L. L.; Janssen, I. C. J. M.; Geurts, J. J. M.; van der Welle, M. E. W.; van Katwijk, M. M.; van der Heide, T.; Roelofs, J. G. M.; Smolders, A. J. P. Sulfide as a soil phytotoxin-a review. *Front. Plant Sci.* **2013**, *4*, 1–14.

(91) Jäckel, U.; Russo, S.; Schnell, S. Enhanced iron reduction by iron supplement: a strategy to reduce methane emissions from paddies. *Soil Biol. Biochem.* **2005**, *37*, 2150–2154.

(92) Ali, M. A.; Oh, J. H.; Kim, P. J. Evaluation of silicate iron slag amendment on reducing methane emission from flood water rice farming. *Agric., Ecosyst. Environ.* **2008**, *128*, 21–26.

(93) Bridgham, S. D.; Cadillo-Quiroz, H.; Keller, J. K.; Zhuang, Q. Methane emissions from wetlands: biogeochemical, microbial, and modeling perspectives from local to global scales. *Global Change Biol* **2013**, *19*, 1325–1346.

(94) Bailey, L. T.; Mitchell, C. P. J.; Engstrom, D. R.; Berndt, M. E.; Coleman Wasik, J. K.; Johnson, N. W. Influence of porewater sulfide on methylmercury production and partitioning in sulfate-impacted lake sediments. *Sci. Total Environ.* **201**7, *580*, 1197–1204.

(95) Ulrich, P. D.; Sedlak, D. L. Impacts of iron amendment on net methylmercury export from tidal wetland microcosms. *Environ. Sci. Technol.* 2010, 44, 7659–7665.

(96) Leverenz, H. L.; Haunschild, K.; Hopes, G.; Tchobanoglous, G.; Darby, J. L. Anoxic treatment wetlands for denitrification. *Ecol. Eng.* **2010**, 36, 1544–1551.

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