Copper availability governs nitrous oxide accumulation in wetland soils and stream sediments

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Abstract

Denitrification is microbiotically-mediated through enzymes containing metal cofactors. Laboratory studies of pure cultures have highlighted that the availability of Cu, required for the multicopper enzyme nitrous oxide reductase, can limit N₂O reduction. However, in natural aquatic systems, such as wetlands and hyporheic zones in stream beds, the role of Cu in controlling denitrification remains incompletely understood. In this study, we collected soils and sediments from three natural environments -- riparian wetlands, marsh wetlands, and a stream -- to investigate their nitrogen species transformation activity at background Cu levels and different supplemented Cu loadings. All of the systems contained solid-phase associated Cu below or around geological levels (40 - 280 nmol g⁻¹) and exhibited low dissolved Cu (3-50 nM), which made them appropriate sites for evaluating the effect of limited Cu availability on denitrification. In laboratory incubation experiments, high concentrations of N₂O accumulated in all microcosms lacking Cu amendment except for one stream sediment sample. With Cu added to provide dissolved concentrations at trace levels (10-300 nM), reduction rate of N₂O to N₂ in the wetland soils and stream sediments was enhanced. A kinetic model could account for the trends in nitrogen species by combining the reactions for microbial reduction of NO₃⁻ to NO₂⁻/N₂O/N₂ and abiotic reduction of NO₂⁻ to N₂. The model revealed that the rate of N₂O to N₂ conversion increased significantly in the presence of Cu. For riparian wetland soils and stream sediments, the kinetic model also suggested that overall denitrification is driven by abiotic reduction of NO₂⁻ in the presence of inorganic electron donors. This study demonstrated that natural aquatic systems containing Cu at concentrations less than or equal to crustal abundances may display incomplete reduction of N₂O to N₂ that would cause N₂O accumulation and release to the atmosphere.

Keywords: wetlands, hyporheic zone, denitrification, nitrous oxide, copper, bioavailability, organic carbon
1. Introduction

Nitrous oxide (N\textsubscript{2}O) is a potent greenhouse gas whose global warming potential per unit mass is 265–298 times that of carbon dioxide (CO\textsubscript{2}) for a 100-year timescale (IPCC, 2014; Sovacool et al., 2021). Global N\textsubscript{2}O emissions in the decade between 2007-2016 averaged 17 Tg N yr\textsuperscript{-1}, of which 57% (9.7 Tg N yr\textsuperscript{-1}) were from natural soils and oceans and ~22% (3.8 Tg N yr\textsuperscript{-1}) were from agricultural soils (Tian et al., 2020).

Denitrification, an anoxic process in which nitrate (NO\textsubscript{3}\textsuperscript{-}) is reduced to N\textsubscript{2}, is a key biogeochemical process that regulates the amount of N\textsubscript{2}O released from both terrestrial and aquatic ecosystems into the atmosphere (Makowski, 2019; Tian et al., 2020; Martinez-Espinosa et al., 2021). Natural aquatic systems, especially those that display vertical redox gradients, such as wetlands and hyporheic zones in streams, are active sites for denitrification (Merill and Tonjes, 2014; Nag et al., 2017; Mwagona et al., 2019; Martinez-Espinosa et al., 2021).

Oxic regions above redox transition zones favor the oxidation of ammonia (NH\textsubscript{3}) to NO\textsubscript{3}\textsuperscript{-} via nitrification, and the anoxic regions below redox transition zones promote denitrification with NO\textsubscript{3}\textsuperscript{-} being reduced to nitrite (NO\textsubscript{2}\textsuperscript{-}), nitric oxide (NO), N\textsubscript{2}O, and N\textsubscript{2}. The incomplete conversion of NO\textsubscript{3}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-} to N\textsubscript{2} causes N\textsubscript{2}O to be released from the aquatic systems to the atmosphere (Twining et al., 2007; Giannopoulos et al., 2020).

An array of metalloenzymes that contain Fe, Cu, and Mo are involved in reducing nitrate and intermediate species to N\textsubscript{2} during denitrification (Godden et al., 1991; Bertero et al., 2003; Nojiri et al., 2007). The transformation of NO\textsubscript{3}\textsuperscript{-} to NO\textsubscript{2}\textsuperscript{-} is catalyzed by respiratory nitrate reductase (Nar), which requires Fe and Mo for complete conversion (Bertero et al., 2003; Jormakka et al., 2004). Depending on the type of microorganism, reduction of NO\textsubscript{2}\textsuperscript{-} to NO is catalyzed by either an iron-containing nitrite reductase (NirS) or a Cu-containing nitrite reductase (NirK) (Godden et al., 1991; Nojiri et al., 2007). NO is rapidly transformed to N\textsubscript{2}O with an Fe-bearing nitric oxide reductase (cNOR), and in the final step, N\textsubscript{2}O is reduced to N\textsubscript{2} by a Cu-rich nitrous oxide reductase (Nos) (Brown et al., 2000).

A scarcity of available Cu can limit the conversion of N\textsubscript{2}O to N\textsubscript{2}. Laboratory studies of pure cultures have demonstrated that Cu limitation resulted in N\textsubscript{2}O accumulation (Iwasaki et al., 1980; Granger and Ward,
Granger and Ward (2003) conducted a study with *Pseudomonas stutzeri* and *Paracoccus denitrificans* in an artificial seawater medium to evaluate the effect of Cu on denitrification. They observed that Cu concentrations of approximately 3 nM caused N$_2$O to accumulate, whereas 10 nM Cu resulted in increased growth rates and complete conversion of N$_2$O to N$_2$. The growth of denitrifying microorganisms, *Alcaligenes* sp. NGIB 11015, *Alcaligenes faecalis* IAM 1015, and *P. stutzeri* was also stimulated by the addition of Cu in the range of 0.5 to 80 µM (Iwasaki et al., 1980; Black et al., 2016).

In soils and sediments, high concentrations of Cu can inhibit denitrification, whereas low availability of Cu can limit microbial activity causing accumulation of intermediate nitrogen species. In estuarine sediments, the addition of 79 µg g$^{-1}$ Cu inhibited microbial activity by 85% and specifically increased the accumulation of NO$_3^-$ and N$_2$O (Magalhaes et al., 2007). Similarly, the addition of Cu at high loadings of 250-1000 µg g$^{-1}$ increased N$_2$O emissions from soils and wetland sediments (Sakadevan et al., 1999; Shaaban et al., 2019). In these three studies, the associated dissolved Cu concentrations were not measured. While the three studies just noted had increased accumulation of N$_2$O associated with high Cu, two other studies found that the addition of Cu to systems with initially low Cu decreased N$_2$O accumulation. A recent study of freshwater wetland sediments that initially had 37.8 µg g$^{-1}$ Cu and were amended with CuSO$_4$ to have 26 µM dissolved Cu showed an increased abundance of nitrite and nitrous oxide reductase genes that enhanced the conversion of N$_2$O to N$_2$ (Giannopoulos et al., 2020). Similarly, a study of freshwater sediments collected from central Indiana also showed that N$_2$O accumulation decreased when the sediments were amended with 50-100 µg g$^{-1}$ Cu (Jacinthe and Tedesco, 2009). On the other hand, the addition of 100 µg L$^{-1}$ Cu did not have any effect on N$_2$O emissions release in freshwater wetland sediments (Doroski et al., 2019). Copper concentrations in uncontaminated environments are typically low (Black et al., 2011; Black et al., 2016), and hence limited Cu bioavailability in such settings may significantly affect N$_2$O conversion via denitrification.

In natural aquatic systems, the relationship between the total Cu amount in the sediments and the bioavailable concentration of dissolved Cu is controlled by the presence of solid phases, such as sulfide minerals, particulate organic carbon, iron and manganese oxyhydroxides, and clay minerals (Du Laing et
changes in aquatic phase parameters, including pH, redox potential, and the concentration of ligands, can mobilize/immobilize metals from solid phases, thus affecting their bioavailability (Du Laing et al., 2009; Zhang et al., 2014). Dissolved Cu is present as a free hydrated ion (Cu^{2+}) and complexes with hydroxides, inorganic ligands (carbonates and chlorides), and organic ligands (humic and fulvic acids) depending on the water composition (Kozelka and Bruland, 1998). Strong organic Cu-chelates, such as complexes with humic acids and fulvic acids, are inert and hence not readily bioavailable, but inorganic hydroxy and carbonate complexes are labile and might be toxic to some microorganisms at concentrations as low as 10 µg L^{-1} (Allen and Hansen, 1996; Bruland et al., 2000; Huang and Wang, 2003; Bourgeault et al., 2013). Several studies have reported that Cu has a high affinity for dissolved organic carbon and exists predominantly as Cu-organic ligand complexes in natural waters (Skrabal et al., 2000; Chakraborty et al., 2015; Ren et al., 2015; Waska et al., 2019). Thus, even dissolved Cu concentrations that would be expected to be optimal for N_2O conversion (>10 nM) might exert a limiting effect on denitrification in natural environments if the Cu is predominantly complexed in non-bioavailable forms.

In natural environments, nitrogen cycling can also occur via processes other than biological denitrification. These processes include dissimilatory nitrate reduction to ammonium (DNRA), anaerobic ammonium oxidation (anammox), chemoautotrophic denitrification, and abiotic photochemical and thermochemical processes (Burgin and Hamilton, 2007; Doane, 2017; Martinez-Espinosa et al., 2021). Other elemental cycles, such as those of iron and sulfur, play important roles in nitrogen cycling in natural environments. Autotrophic and mixotrophic denitrifiers can gain energy from mediating the reduction of NO_3^{-} by inorganic electron donors that include sulfur compounds (S^{0}, S^{2-}, S_2O_3^{2-}), pyrite (FeS_2), Fe^{2+}, Fe^{0}, and Mn^{2+} (Davidson et al., 2003; Weber et al., 2006; Melton et al., 2014; Di Capua et al., 2019; Wei et al., 2019). Additionally, abiotic reduction of NO_3^{-}/NO_2^{-} by dissolved Fe(II) or Fe(II)-bearing minerals has been studied extensively as a pathway for N_2O or N_2 formation under anoxic conditions (Moraghan and Buresh, 1977; Ottley et al., 1997; Matocha et al., 2012; Melton et al., 2014; Peters et al., 2014; Buchwald et al., 2016; Liu et al., 2019; Matus et al., 2019; Chen et al., 2020). Fe(II)-rich flooded soils showed decreased
N$_2$O emissions because high Fe(II) concentrations favored the complete conversion of NO$_3^-$ to N$_2$ (Wang et al., 2016). A recent study of marine sediments found that ~15-25% of the total N$_2$O released was produced by abiotic nitrite reduction in the presence of Fe(II) (Otte et al., 2019).

The effects of redox conditions, substrate availability, temperature, and pH on denitrification have been widely studied (Nowicki, 1994; Koponen et al., 2004; Baeseman et al., 2006; Ward et al., 2008; Nag et al., 2017). There has also been significant progress in understanding the toxic effects of elevated Cu levels on the denitrification pathway, but only limited studies have investigated the effect of Cu availability on N$_2$O accumulation in uncontaminated aquatic systems (Twining et al., 2007; Giannopoulos et al., 2020). A broader understanding of how Cu affects N-cycling in natural systems can improve the accuracy of ecosystem models, such as the Dynamic Land Ecosystem Model (DLEM) (Tian et al., 2020), used to estimate N$_2$O emissions. The objectives of this study were to (1) investigate the effect of trace concentrations of dissolved Cu on nitrate reduction and the formation of reaction products (NO$_2^-$ and N$_2$O) with soils and sediments from three different natural aquatic systems and (2) develop a kinetic model for the denitrification reactions that can quantify the effect of Cu addition on the transformation and accumulation of nitrogen species in environmental systems.

2. Materials and methods

2.1 Description of sites

To investigate the effect of Cu on denitrification, we selected three separate natural aquatic systems: marsh wetlands at Argonne National Laboratory (ANL) in Lemont, Illinois; riparian wetlands in the Tims Branch (TB) watershed at the Savannah River Site in Aiken County, South Carolina; and East Fork Poplar Creek (EFPC) at Oak Ridge National Laboratory (ORNL) in Oak Ridge, Tennessee. Detailed information on the sampling sites, sampling techniques, and soil characterization can be found in our recent study on trace metal speciation at these sites (Yan et al., 2021). Briefly, soil/sediment and surface water samples were collected from two different locations at each aquatic system. To identify locations within the sites, we used
the labels “Riparian 1” and “Riparian 2” for Tims Branch riparian wetland soils; “Stream 1” and “Stream 2” for Oak Ridge stream sediment sites, and “Marsh 1” and “Marsh 2” for Argonne marsh wetland soils.

2.2 Sampling and characterization

Soil or sediment cores were collected in polycarbonate tubes. Cores from the marsh wetland and riparian wetland were shipped on ice to Washington University in St. Louis, where they were extruded from the tubes and immediately transferred to an anaerobic chamber (Coy Laboratory Products, 3% H\textsubscript{2}/97% N\textsubscript{2}, with Pd catalyst) to maintain anaerobic conditions. The cores from the stream sediment site were extruded at ORNL within 1 h of sampling, impulse sealed in polyethylene pouches in an anaerobic chamber, and stored in a refrigerator at 4°C before being shipped on ice to Washington University, where they were stored in an anaerobic chamber.

Surface water samples were filtered using 0.22 µm mixed cellulose ester (MCE) syringe filters. A portion was immediately acidified to 2% nitric acid (HNO\textsubscript{3}), and the rest of the filtered water was stored at 4 °C prior to anion and nutrient analysis. The acidified surface water samples were analyzed to quantify the dissolved major elements (sodium, magnesium, aluminum, silicon, potassium, and calcium) and trace metals (cobalt, nickel, copper, and zinc). The major elements were quantified using a Thermo Scientific iCap 7400 Duo inductively-coupled plasma optical emission spectrometer (ICP-OES), and the trace metals were quantified with an inductively coupled plasma mass spectrometer (PerkinElmer Elan DRC II). The unacidified water samples were used to measure the concentrations of dissolved anions (Br\textsuperscript{-}, Cl\textsuperscript{-}, F\textsuperscript{-}, and SO\textsubscript{4}\textsuperscript{2-}) using a Thermo Scientific Dionex Integraion high-pressure ion chromatograph (IC) with a conductivity detector. The major elements and trace metals were extracted from the soils/sediments by microwave-assisted digestion and were analyzed using ICP-OES and ICP-MS, respectively (Yan et al., 2021). The extractable nutrients (nitrate, ammonium, and phosphate) in the soils and sediments were obtained using a 2 M potassium chloride extraction method adapted from previous studies (Sparks et al., 1996; Pansu and Gautheyrou, 2006) and were measured using a Seal Analytical AQ300 Discrete Multi-Chemistry Analyzer.
2.3 Reagent preparation

All the solutions were prepared in an anaerobic chamber (3% \( \text{H}_2/97\% \text{N}_2 \), with Pd catalyst) using deoxygenated deionized water (>18.2 MΩ cm). Deoxygenated water was prepared by bubbling \( \text{N}_2 \) gas through it for 4-5 h, followed by bubbling the water in an anaerobic chamber for 3 h with a filtered stream of the anaerobic chamber atmosphere that had been passed in sequence through solutions of ferrous chloride and KOH to remove traces of oxygen and carbon dioxide. A colorimetric assay (CHEMets test kit K-7511) was used to ensure that the dissolved oxygen level in the deoxygenated water was below the detection limit (2 \( \mu \text{g/L} \)). Copper chloride dihydrate (99.99%, Sigma Aldrich) was used to vary the Cu loading in denitrification studies. All other salts (sodium chloride, potassium chloride, sodium sulfate, magnesium chloride hexahydrate, magnesium sulfate, calcium sulfate dihydrate, ammonium chloride, and disodium phosphate) used for preparing simulated site water were purchased from Thermo Fisher Scientific and were of reagent grade. Sodium nitrate and potassium nitrate (99.9%, Sigma Aldrich) were used for preparing a stock solution of nitrate. Nitric acid (trace metal grade, Thermo Fisher Scientific) was used to acidify samples for dissolved metal analysis. Reagents and calibration standards for nutrient analysis were prepared using reagent grade chemicals.

2.4 Laboratory incubation experiments

The incubation studies were conducted with the samples from the riparian wetlands (Riparian 2 and Riparian 1), the stream (Stream 2 and Stream 1) and the marsh wetlands (Marsh 1). The two locations in riparian wetland soils and stream sediments had different total carbon, sulfur, and metal contents and exhibited different solid-phase speciation of Cu. Both the locations in the marsh wetland soils showed similar characteristics (Yan et al., 2021), so the samples from only one marsh wetland location (Marsh 1) were used in further studies. Cu uptake experiments were performed to determine the Cu concentration range to consider in the incubation studies. The soils and sediments were completely homogenized before uptake experiments. These experiments were conducted in 15 mL polypropylene tubes containing 1-200 \( \mu \text{M} \text{CuCl}_2 \) and 10 mL of simulated site water, maintained at the desired pH (5.0 for Riparian 1 and Riparian...
2, 7.6 for Stream 1 and Stream 2, and 7.0 for Marsh 1) and 0.5 g of soil/sediment. The recipe for the simulated water included the major cations and anions analyzed in the water samples (Table S1 in the Supplementary material). Samples were rotated end-over-end for 24 h to ensure complete mixing. After 4 h and 8 h of rotation, the pH values were readjusted to the original values, using 1M NaOH and 2M HCl solutions. The suspension was then immediately filtered using disposable 0.22 µm MCE syringe filters and acidified to 1% HNO₃. Dissolved Cu concentrations were measured by ICP-MS (PerkinElmer, NexION 2000).

Incubation experiments were initiated under anaerobic conditions in 100 mL serum bottles containing 2.5 g of homogenized soil/sediment along with 50 mL of simulated site water. The simulated water only contained major cations and anions and lacked trace metals or Fe and Mn (Table S1). The pH of the slurries was adjusted to 5.0 for Riparian 1 and Riparian 2, 7.6 for Stream 1 and Stream 2, and 7.0 for Marsh 1, using NaOH and HCl solutions. For each site, three different conditions were studied: no Cu added (control), low Cu loading, and high Cu loading (loading details are in Table 1). The different Cu loadings were selected based on the Cu uptake experiments discussed above. The selection of loadings was done so that the dissolved concentrations after 24 h of equilibration ranged between 10-100 nM and 100-2500 nM for low and high Cu loadings, respectively (Figure 1). After 24 h of equilibration, 1 mM NO₃⁻ was added, and the bottles were sealed with a 20 mm butyl stopper and aluminum cap. Immediately after the addition of NO₃⁻, 1 mL of the fluid was sampled to determine the concentrations of dissolved metals and nutrients. The bottles were removed from the anaerobic chamber after NO₃⁻ addition and were flushed for 10 minutes with ultrapure N₂ to remove trace amounts of O₂ and H₂ from the headspace.

To determine N₂O concentrations, headspace gas samples were taken from each serum bottle at 24 h intervals and were transferred to 3 mL pre-evacuated glass vials (Exetainer®, Labco, United Kingdom) using a gas-tight syringe. The vials were stored upside down to prevent gas leakage from the septum. To separate the dissolved phase, 1 mL of slurry from each serum bottle was sampled and centrifuged (Spectrafuge 16M) for 5 min at 5000 rpm. The supernatant obtained was divided into three parts, 300 µL was acidified to 1% HNO₃ for dissolved metal analysis, 400 µL was stored for nutrient analysis, and the
remaining supernatant was used to estimate the pH using Whatman pH indicator strips. At the end of incubation experiments, the final pH value was recorded using a pH electrode. The water was filtered and stored at 4°C to determine the dissolved organic carbon (DOC) concentration at the end of incubation experiments.

**2.5 Analytical techniques used**

N₂O concentrations in the samples were measured using a gas chromatograph (GC) (Thermo Scientific GC TRACE 1310). Specifically, 1000 µL of the gas sample was injected (split injection at the split rate of 10:1) into the GC inlet (heated to 130 °C) using a TriPlus RSH (Thermo Scientific) autosampler equipped with a 2500 µL headspace syringe. The temperature of the column (Supelco Carboxen 1010 PLOT, 30 m x 0.32 mm) was maintained at 50 °C for 7.5 min, after which it was ramped to 130 °C using a rate of 20 °C/min and then kept at this temperature for 2 min. Helium was used as the carrier gas at a flow rate of 30 mL/min.

A pulse discharge detector (PDD) at 150 °C was used for the analysis of N₂O. The concentration of the standards varied from 10 ppmv to 0.1% N₂O and were prepared using a certified gas standard from Airgas. The concentration of N₂O dissolved in the fluid was determined using the ideal gas law and Henry’s gas solubility law. The value of Henry’s constant at 25 °C used for determining dissolved N₂O was 2.4*10⁻⁴ mol m⁻³ Pa⁻¹ (Sander, 2015). The total N₂O present in the microcosm at the time of sampling was the sum of the gas in the headspace and the gas dissolved in the water.

The dissolved metal concentrations (Cu, Fe, and Mn) were measured using inductively coupled plasma mass spectrometer (PerkinElmer Elan DRC II). DOC concentrations were determined using a total organic carbon analyzer (Shimadzu TOC-L). The nutrient concentrations (NO₃⁻, NO₂⁻ and NH₄⁺) were obtained spectrophotometrically using a discrete multi-chemistry analyzer (Seal Analytical AQ300). The samples used for nutrient analysis were frozen and then thawed overnight at 4°C before analysis. Nitrite was measured by the reaction of the sample with sulfanilamide in dilute phosphoric acid to form a diazonium compound which binds to N-(1-naphthyl)-ethylenediamine dihydrochloride to form an azo dye detected at 520 nm (Huffman and Barbarick, 1981). To determine the nitrate concentration, NO₃⁻ was first
reduced to NO$_2^-$ by cadmium and then the NO$_2^-$ was measured. Ammonium present in the samples was
determined by reacting the samples with hypochlorite liberated from dichloroisocyanurate in an alkaline
solution followed by a reaction with salicylate in the presence of nitroferricyanide to form a blue indophenol
dye, which was measured at 660 nm (Krom, 1980).

2.6 Complexation of dissolved Cu by DOC

To estimate the speciation of Cu in the presence of organic carbon in the incubation experiments, the
nonideal competitive adsorption-Donnan (NICA-Donnan) model in Visual MINTEQ 3.1 (Yan and Korshin,
2014) was used. The model is a combination of NICA which enables simulation of metal complexation to
humic substances, and a Donnan model describing nonspecific electrostatic interactions between ions and
humic substances (Benedetti et al., 1995; Benedetti et al., 1996; Ren et al., 2015). Although humic
substances might not truly represent the dissolved organic matter present in aquatic systems (Kleber and
Lehmann, 2019; Myneni, 2019), we used the NICA-Donnan model to estimate the aqueous speciation of
Cu because this model has previously provided accurate predictions of metal speciation and availability in
natural systems (Han et al., 2014; Ponthieu et al., 2016). NICA considers competitive binding between
protons and metals to humic substances by accounting for binding site heterogeneity and ion-specific
nonideality (Benedetti et al., 1995). The generic parameters obtained in previous studies for Cu and proton
binding to humic material were used (Milne et al., 2001; Milne et al., 2003; Xu et al., 2016). Water
chemistry parameters (pH, total dissolved elements (Table S1)), dissolved Cu, Fe, and Mn, and dissolved
organic carbon (DOC) were used as the input parameters for determining dissolved Cu speciation. Three
sets of conditions were used to account for Cu speciation at different total dissolved Cu concentrations
corresponding to the control, low Cu loading, and high Cu loading experiments. The combined NICA-
Donan model provides the amount of Cu bound to humic substances by using equations S1 and S2 shown
in supplementary material (details of the methodology and parameters used are provided in Section S1 and
Table S3). Under anoxic environments, as in our incubation experiments, Cu(II) can be reduced to Cu(I)
by microorganisms, inorganic reductants, and redox-active functional groups on dissolved organic matter.
Cu(I) can form stable complexes with inorganic ligands or thiol groups of organic matter (Yuan et al., 2012; Fulda et al., 2013b; Fulda et al., 2013a). In our speciation calculations, we have not accounted for the formation of these Cu(I)-thiol complexes, and dissolved Cu is assumed to be in Cu(II) form.

### 2.7 Kinetic model

A kinetic model was developed to quantify the effect of Cu on denitrification. Michaelis-Menten expressions were used to describe the evolution of NO$_3^-$, NO$_2^-$, N$_2$O, and N$_2$ during denitrification (Eq 1-4).

\[
\frac{d[CNO_3^-]}{dt} = -V_{\text{max}} \frac{[CNO_3^-]}{K_{NO_3} + [CNO_3^-]} \tag{1}
\]

\[
\frac{d[NO_2^-]}{dt} = V_{\text{max}} \left( \frac{[CNO_2^-]}{K_{NO_2} + [CNO_2^-]} - \frac{[CNO_2^-]}{K_{NO_2} + [CNO_2^-]} \right) - k_{ab}[CNO_2^-] \tag{2}
\]

\[
\frac{d[N_2O]}{dt} = V_{\text{max}} \left( \frac{[CNO_2^-]}{K_{NO_2} + [CNO_2^-]} - \frac{[CN_2O]}{K_{N_2O} + [CN_2O]} \right) \tag{3}
\]

\[
\frac{d[N_2]}{dt} = V_{\text{max}} \left( \frac{[CN_2O]}{K_{N_2O} + [CN_2O]} \right) + k_{ab}[CNO_2^-] \tag{4}
\]

Here, $V_{\text{max}}$ denotes the maximum reaction rate under unlimited substrate supply (mmol-N L$^{-1}$ day$^{-1}$), $C_y$ are the concentrations of N-containing species (mmol-N L$^{-1}$), and $K_y$ values are Michaelis-Menten parameters (mmol-N L$^{-1}$) describing the substrate concentration at which the reaction rate is half $V_{\text{max}}$ (Bowman and Focht, 1974; Kremen et al., 2005). For model development, the concentration of N$_2$ was calculated from the mass balance on nitrogen species. As discussed earlier, abiotic nitrite reduction to N$_2$ by inorganic electron donors, such as inorganic sulfur compounds (S$^\circ$, S$^2$-, S$_2$O$_3^{2-}$), pyrite (FeS$_2$), thiocyanate (SCN$^-$), and ferrous ion (Fe$^{2+}$), is a pathway in N-cycling (Zhu and Getting, 2012; Zhu-Barker et al., 2015; Di Capua et al., 2019; Otte et al., 2019). Therefore an additional reaction, accounting for the abiotic reduction of NO$_2^-$ to N$_2$, was included in the model. A pseudo first-order reaction was used to define the abiotic reduction of NO$_2^-$ in the system (incorporated in Eq 2 and 4), where $k_{ab}$ (day$^{-1}$) is the pseudo first-
order rate constant assuming that the reductants are in excess (Matocha et al., 2012; Chen et al., 2020), and $C_{NO_2}$ is the concentration of $NO_2^-$ in the dissolved phase. Using the ODE45 function in MATLAB R2018a (Shampine et al., 2003; Anyigor and Afiukwa, 2013), the unknown parameters were calculated. We optimized the parameters and obtained the error estimates by minimizing the residuals using a non-linear least-squares method. The value of $V_{max}$ was determined using Eq 1 and data on the change in nitrate concentration with time, and then the value was fixed to determine the rate parameters defined in Eq 2-4. A constant value of $V_{max}$ can be employed when organic carbon is present in excess of $NO_3^-$ (Kremen et al., 2005). The total organic carbon present in the systems studied far exceeded the amount of $NO_3^-$ added (Section S2 in SM). In separate optimations in which we allowed the values of $V_{max}$ to be different in equations 1-4, the values were all in the narrow range of range $0.25 – 0.5 \text{ mmol-N L}^{-1} \text{ day}^{-1}$.

3. Results

3.1 Characterization of soils and sediments

Both the surface water samples, and the soils and sediments were characterized to determine their total carbon, sulfur, metals, and nutrient concentrations. The detailed results are presented in our recent study focused on trace metal micronutrient speciation in wetland soils and stream sediments (Yan et al., 2021). The surface water in the marsh wetlands (Marsh 1) and stream sediments (Stream 1 and Stream 2) had pH values ranging from 7.5-8.1, and they contained high concentrations of calcium, magnesium, and sulfate. However, the riparian wetland surface water samples (Riparian 1 and Riparian 2) were at pH 5.5-6.0 with substantially lower concentrations of major elements.

The mineralogy at all of the studied sites is dominated by quartz, with variations in minor phases (Yan et al., 2021). The total organic carbon content of aquatic systems varies with location: the marsh wetland site (Marsh 1) contained the highest carbon content (9.0%), whereas Stream 2 exhibited the lowest carbon content (0.5%) (Table S2). The sulfur content was low at all sites (below 0.24%), following the trend Marsh 1 > Riparian 2 ≈ Stream 1 > Riparian 1 ≈ Stream 2. The total Fe concentration in the solid phases was similar at all the sites (200 to 460 µmol/g), except for Riparian 1, which contained an order of magnitude
less Fe than the other soils/sediments. The concentration of Mn was two to three orders less than the Fe content, with the highest values observed in stream sediments. Extractable ammonium in soils/sediments was greater than extractable NO$_3^-$ and NO$_2^-$ at all the locations, which may indicate denitrification and/or ammonium retention via cation exchange (Table S2).

### 3.2 Selection of Cu loadings

The total solid-phase concentrations of Cu were well below the crustal abundance (428 ± 61 nmol/g) (Rudnick and Gao, 2003) at all the studied sites. The marsh wetland soil (Marsh 1) and Riparian 2 location in the riparian wetlands contained higher concentrations of solid-phase Cu than the other locations. The dissolved Cu concentrations were less than 50 nM at the selected locations (Table 1), indicating that Cu at these sites is predominantly associated with mineral and organic solid phases and might not be bioavailable to microorganisms for N-cycling. Cu uptake experiments were used to determine the Cu loadings to use in incubation experiments to target particular dissolved Cu concentrations (Figure 1). The selected loadings resulted in dissolved Cu concentrations in the range of 15-300 nM and 50-2300 nM for low and high Cu amendments, respectively in the incubation experiments (Table 1).

### 3.3 Effect of Cu on evolution of nitrogen species concentrations during incubation

The controls and the low-Cu amended sets showed similar NO$_3^-$ reduction profiles in all the systems studied (Figure 2). In controls and low-Cu amended systems, the complete reduction of 1 mM added NO$_3^-$ occurred within 16, 5, and 6 days for Riparian 2, Stream 1, and Marsh 1, respectively. In Riparian 1 and Stream 2 experiments, complete reduction of NO$_3^-$ did not occur, even after 27 days and 20 days of incubation, respectively. A small delay in NO$_3^-$ reduction after Cu addition was observed at high Cu loading in Riparian 1, Riparian 2, and Stream 2 incubation experiments (Figure 2a, 2e, and 2m).

The presence of detectable NO$_2^-$ was transient and showed a brief appearance followed by a rapid decline in concentration in Riparian 2, Stream 1, and Marsh 1 incubations. In Riparian 1 soils, the dissolved
NO$_2^-$ concentrations were below the detection limit (0.0005 mmol-N L$^{-1}$) throughout the experiment, suggesting rapid conversion of NO$_2^-$ in these soils (Figure 2b). In the case of Riparian 2 and Stream 2 systems, Cu addition affected NO$_2^-$ formation/reduction because more NO$_2^-$ was detected in controls as compared to Cu-amended sets.

For all the systems studied, less N$_2$O transiently accumulated in the sets amended with Cu. We did not observe persistent accumulation of N$_2$O in the case of Riparian 1 soils (Figure 2c), however, the maximum concentration of N$_2$O (control: 0.040 mmol-N L$^{-1}$ at 14 days, low-Cu: 0.032 mmol-N L$^{-1}$ at 10 days, and high-Cu: 0.021 mmol-N L$^{-1}$ at 12 days) decreased as the dissolved Cu concentration increased. For Riparian 1 controls and low-Cu amended microcosms, N$_2$O started to accumulate after 3 days of incubation, whereas in high-Cu amended sets, N$_2$O accumulation was only observed after 6 days of incubation. The complete reduction of N$_2$O to N$_2$ was fast in low-Cu amended Riparian 1 sets; we did not observe N$_2$O after 14 days in low-Cu added sets, whereas it took 29 and 23 days to completely reduce N$_2$O in high-Cu added and control sets, respectively. In the Riparian 2 control, N$_2$O accumulated in the headspace and persisted until the end of the experiment at 30 days, whereas the N$_2$O concentration first increased and then decreased after 10 days and 16 days in Riparian 2 sets amended with low Cu and high Cu, respectively (Figure 2g).

For Riparian 2, relative to controls, the maximum N$_2$O concentration decreased by 38.6% in low Cu-added sets and by 70.1% in high Cu-added sets. In the case of the stream sediments, Stream 1 showed a significant effect of Cu addition on N$_2$O reduction; with respect to controls, the peak N$_2$O concentration decreased by 2.6 times and 7.8 times in sets with low and high Cu loading, respectively (Figure 2k). In Stream 2 and Marsh 1 systems, we were able to measure detectable N$_2$O in the headspace of only the controls; in the Cu-amended sets, any N$_2$O generated was rapidly reduced before the N$_2$O reached detectable levels (Figure 2n and 2r).

The concentration of dissolved ammonium (NH$_4^+$) remained relatively constant throughout the experiment for all the treatments (control, low loading, and high loading) in the systems studied (Figure 2). Dissolved NH$_4^+$ was highest in Marsh 1, at 1.039 ± 0.048 mmol-N/L. Riparian 2 and Riparian 1, contained 0.130 ± 0.004 mmol-N/L and 0.033 ± 0.004 mmol-N/L NH$_4^+$, respectively. The dissolved NH$_4^+$
concentrations in stream sediments averaged 0.053 ± 0.005 mmol-N/L and 0.172 ± 0.019 mmol-N/L in the Stream 1 and Stream 2 samples, respectively.

3.4 Variation in dissolved metal (Cu, Fe, and Mn) concentration

Dissolved Cu, Fe, and Mn were monitored throughout the incubation experiments (Figure 3). The dissolved Cu concentrations in the unamended control microcosms followed the trend Marsh 1 > Riparian 2 > Riparian 1 > Stream 2 > Stream 1. The dissolved Cu concentration remained relatively constant throughout the experiment for Riparian 2, Stream 2, and Stream 1 experiments. However, a decrease in Cu concentration was observed in all Riparian 1 sets, and controls and low-Cu amended Marsh 1 sets in the initial days of incubation (Figure 3d and 3m).

A decrease in dissolved Fe concentration was observed during the experiment for the riparian wetland soils (Riparian 1 and Riparian 2) and stream sediments (Stream 1 and Stream 2), whereas, in marsh wetland soil (Marsh 1), dissolved Fe concentration did not fluctuate during the 8 days of incubation (Figure 3). In the Riparian 1 and Riparian 2 experiments, the dissolved Mn concentration remained constant and was similar for all treatments (control, low and high Cu-loadings). However, in Stream 1 and Marsh 1, we observed a decrease in the concentration of Mn until 7 days and 4 days, respectively and then it remained constant (Figure 3i and 3o). Mn concentrations in Stream 2 experiments amended with high Cu were greater than the concentrations in controls and low Cu-loading experiments (Figure 3l).

3.5 Effect of Cu addition on denitrification rate

The effect of Cu on denitrification was quantified with the help of the kinetic model. We obtained Michaelis-Menten parameters (Table 2) and the abiotic rate constant for the set of differential equations defined earlier (Eq 1-4). Here, \( K_{\text{NO}_3^-} \), \( K_{\text{NO}_2^-} \), and \( K_{\text{N}_2\text{O}} \) values reflect the ability of the microbial community present in the soils and sediments to reduce \( \text{NO}_3^- \) to \( \text{NO}_2^- \), \( \text{NO}_2^- \) to \( \text{NO} \), and \( \text{N}_2\text{O} \) to \( \text{N}_2 \), respectively under the conditions studied. NO is rapidly transformed to \( \text{N}_2\text{O} \), hence the conversion of NO to \( \text{N}_2\text{O} \) was assumed to not be rate-limiting. The rate constant for abiotic reduction of \( \text{NO}_2^- \) to \( \text{N}_2 \) by inorganic donors in the
system is defined by $k_{ab}$, and inclusion of this reaction helped us reproduce the major features of all the experiments (Figure 4). The abiotic reduction of NO$_2$ to N$_2$O is also a possible pathway, but the incorporation of this reaction into the kinetic model did not improve the fit to experimental data. Michaelis-Menten parameters have an inverse relationship with rates, unlike rate constants; the smaller the value of $K_y$, the faster the forward reaction; whereas the greater the value of $k_{ab}$, the faster the rate of abiotic nitrite reduction. The model was able to describe the major features for nitrogen species reduction at all the sites except for Marsh 1. For the Marsh 1 site, we observed a lag in NO$_3$- reduction in all the incubation experiments. Our model does not account for the acclimatization time of microorganisms after NO$_3$ addition which could have caused poor fitting of data from the Marsh 1 experiments.

The modeled $K_{NO_3}$ values show that NO$_3$- reduction is fastest in Stream 1 sediments followed by Marsh 1, Riparian 2, Riparian 1, and Stream 2. The parameter $K_{NO_3}$ was similar for control and low Cu-loading in all the systems studied. However, the modeled value increased (Table 2) in high Cu-loading sets initiated with Riparian 1, Riparian 2, and Stream 2 sediments, indicating that the reduction of NO$_3$- to NO$_2$- is slower in these sets amended with a high concentration of Cu.

Cu addition decreased NO$_2$- accumulation in Riparian 2 and Stream 2 samples. The value of $K_{NO_2}$ was less in Cu-amended Riparian 2 experiments (control: 0.68 mmol-N L$^{-1}$; low Cu: 0.22 mmol-N L$^{-1}$; and high Cu: 0.33 mmol-N L$^{-1}$), which signified that Cu enhanced the rate of NO$_2$- reduction in Riparian 2 soils. Similarly, in Stream 2 sediments, the modeled $K_{NO_2}$ values show a substantial decrease in Cu-amended sets (control: 0.0.079 mmol-N L$^{-1}$; low Cu: 0.0067 mmol-N L$^{-1}$; and high Cu: 0.0035 mmol-N L$^{-1}$).

The rate of N$_2$O to N$_2$ conversion, as indicated by the $K_{N_2O}$ parameter, increased upon Cu addition in the Riparian 1, Riparian 2, Stream 1, and Stream 2 locations. In the marsh wetland soil, $K_{N_2O}$ remained relatively constant in the control and Cu-amended experiments. The effect of Cu addition on N$_2$O reduction was greatest in the Riparian 1 and Riparian 2 soils; in Riparian 2 soils, the value of $K_{N_2O}$ decreased from 11000 mmol-N L$^{-1}$ in unamended control experiments to 0.48 mmol-N L$^{-1}$ and 0.21 mmol-N L$^{-1}$ in low-Cu loading and high-Cu loading experiments, respectively. Similarly, in Riparian 1 soils, Cu addition increased
the \( \text{N}_2\text{O} \) conversion significantly; \( K_{\text{N}_2\text{O}} \) values decreased from 6900 mmol-N L\(^{-1}\) to 24 mmol-N L\(^{-1}\) and 3.5 mmol-N L\(^{-1}\) at low and high Cu loadings, respectively.

The rate of abiotic NO\(_2^-\) to \( \text{N}_2 \) reduction was greater in Riparian 1 and Riparian 2 wetland soils than in the other three systems; all microcosms incubated with Stream 2 sediments and Marsh 1 soils showed negligible \( k_{\text{ab}} \) values. NO\(_2^-\) was not detected in any Riparian 1 incubation experiments (Figure 2b); the abiotic rate constant for NO\(_2^-\) to \( \text{N}_2 \) reduction is high in Riparian 1 soils, which could have prevented NO\(_2^-\) accumulation in these soil incubations. The values of \( k_{\text{ab}} \) were similar for all the different incubation studies (controls, low-Cu, and high-Cu loading) of a location, signifying that this step is not affected by the presence of Cu.

### 3.6 Labile concentration of Cu in soil/sediment incubations

To understand Cu bioavailability as a nutrient and as a toxic element, we estimated the speciation of dissolved Cu using Visual MINTEQ 3.1 and the NICA-Donnan model at the studied experimental conditions and in the presence of dissolved organic carbon (Figure 5). The calculations predict that DOC substantially decreased the lability of Cu in the systems studied. Here, the labile Cu concentration is defined as the sum of \( \text{Cu}^{2+} \), \( \text{Cu(OH)}^+ \), and \( \text{Cu(OH)}_2(\text{aq}) \). In riparian wetland controls, Riparian 1 and Riparian 2, in the studied pH range (5-6), Cu is predominantly present as Cu-organic matter complexes (Figure 6), and the labile Cu concentration (Table S4), is < 3 nM. Similarly, in Stream 1 controls, the total labile Cu concentration is < 1 nM in the experimental pH range (7.6-9.0). Due to a lower concentration of DOC in Stream 2 (Figure 5), ~80% of dissolved Cu is present as labile Cu in Stream 2 controls. High DOC in Marsh 1 samples complexed ~84% of dissolved Cu(II), and only 7.6 nM remained as labile concentration in the fluid of controls. In all the Cu-amended experiments, the lability of Cu was greater than the optimum concentration (3-10 nM) determined to complete \( \text{N}_2\text{O} \) to \( \text{N}_2 \) conversion in pure culture studies and lake systems (Granger and Ward, 2003; Twining et al., 2007). Additionally, in high-Cu amended experiments, labile Cu in Riparian 1, Riparian 2, and Stream 2 was substantially higher (> 350 nM), which could inhibit

4. Discussion

4.1 Role of labile Cu in nitrogen cycling

The trends in nitrogen species transformation with and without Cu addition varied between different sites as discussed in section 3.3. The labile concentration of Cu rather than the total dissolved concentration helped us understand the evolution of nitrogen species at different sites. This study found that high dissolved Cu concentrations decreased the rate of NO$_3^-$ reduction in the riparian wetland soils (Riparian 1 and Riparian 2) and from the Stream 2 sediments. This observation is in line with previous results: higher concentrations of Cu can inhibit denitrification by causing a shift in the community composition of denitrifiers (Magalhaes et al., 2007; Wang et al., 2018; Zhao et al., 2020). A recent study that focused on evaluating the toxic effects of copper oxide (CuO) nanoparticles on denitrification in soils observed that the Cu ions (Cu$^{2+}$) released upon nanoparticle application can decrease nitrate reductase (Nar) activity by 21.1-42.1%, causing an 11-times decrease in NO$_3^-$ reduction (Zhao et al., 2020). Elevated Cu$^{2+}$ concentrations (> 500 µg g$^{-1}$ in solid-phase and ~0.95 mg L$^{-1}$ in dissolved form) can decrease biological denitrification by inhibiting extracellular or intracellular enzymes (Fu and Tabatabai, 1989; Sakadevan et al., 1999; Ochoa-Herrera et al., 2011). At high Cu loadings in the above-mentioned locations (Riparian 1, Riparian 2, and Stream 2), the labile Cu concentrations estimated using the NICA-Donnan model were higher (> 350 nM) than in Stream 1 (28 nM) and Marsh 1 (150 nM) locations (Table S4), and the higher concentrations could have inhibited NO$_3^-$ reduction during the incubation experiments.

Incomplete reduction of NO$_3^-$ was observed in incubation experiments using Riparian 1 soils and Stream 2 sediments even when Cu was not added and had low labile concentrations. This suggests that denitrification was limited by the low total organic carbon content at these sites (Ward et al., 2008). While the total organic carbon present in the soils/sediments exceeded the amount stoichiometrically required for complete reduction of NO$_3^-$ (Section S2 in SM), not all of the organic carbon will be available to denitrifying
microorganisms (Schmidt et al., 2017). The biodegradability of the organic matter depends upon molecular characteristics of the organic matter; carbohydrates, proteins, and organic acids are easily degradable, whereas, aromatic and hydrophobic organic entities are recalcitrant to microbial activity (Marschner and Kalbitz, 2003). The low degradability of organic matter is probably limiting NO$_3^-$ reduction in Riparian 1 and Stream 2 sites. In future investigations the CO$_2$ respiration rate could be measured during the incubation experiments to assess the association between organic matter oxidation and denitrification.

The dissolved Cu concentration in the Riparian 2 control (41 ± 9 nM) was higher than the optimum concentration required for N$_2$O to N$_2$ conversion in pure culture studies (3 to 10 nM) (Granger and Ward, 2003; Twining et al., 2007; Glass and Orphan, 2012). However, N$_2$O accumulation was observed at the Riparian 2 site, suggesting that the dissolved Cu may not have been completely bioavailable to the microorganisms that convert N$_2$O to N$_2$. The high DOC (47 mg C/L) at Riparian 2 (Figure 5) indicated the presence of soluble organic ligands. These ligands may form soluble complexes with Cu, thus decreasing Cu availability (Du Laing et al., 2009; Zhang et al., 2014). The free Cu$^{2+}$ and the Cu(II)-hydroxo complexes concentrations control the bioavailability of Cu rather than the total dissolved concentration (Black et al., 2011; Bourgeault et al., 2013). The labile Cu concentration in Riparian 2 control experiments, shown as the sum of Cu$^{2+}$, Cu(OH)$^+$, and Cu(OH)$_2$(aq) (Table S4), is ~ 1.4 nM which is less than the optimum Cu concentration (3-10 nM) (Granger and Ward, 2003; Twining et al., 2007; Glass and Orphan, 2012) required for conversion of N$_2$O to N$_2$ in pure culture studies. Thus, the low lability of Cu in Riparian 2 control sets could have caused persistent N$_2$O accumulation in the headspace.

In Riparian 1 soils, both the background dissolved Cu concentration (29.3 nM) and the solid-phase-associated Cu (48.3 nmol/g) were less than the Cu concentration in Riparian 2 soils (dissolved: 41 nM; solid-phase: 262.3 nmol/g), but N$_2$O did not accumulate persistently in the headspace of Riparian 1 soils. This observation suggests that bioavailable Cu for N$_2$O to N$_2$ conversion is more abundant in Riparian 1 soils. Riparian 1 soils have less dissolved organic carbon (23 mg C/L) than Riparian 2 soils, and thus are less able to decrease the bioavailability of Cu by forming soluble complexes of organic matter with Cu. The speciation results corroborated the hypothesis because in the pH range studied, the dissolved labile Cu in
Riparian 1 controls (2.8 ± 0.9 nM) was greater than Riparian 2 controls (1.4 ± 0.8 nM). Prior study on a lake system indicated that the presence of 3 nM dissolved Cu decreased N₂O accumulation during denitrification relative to systems containing no Cu (Twining et al., 2007). Although the rate of N₂O to N₂ conversion was slow in Riparian 1 controls as compared to Cu-amended sets (Table 2), the labile-Cu concentration closer to optimum range (3-10 nM) prevented persistent N₂O accumulation in the headspace.

The concentration of accumulated N₂O in the headspace of Stream 1 controls was greater than the Stream 2 controls. Both stream sediment sites contained low concentrations of dissolved Cu in the control sets (3.1 ± 1 nM at Stream 1, and 6.2 ± 1.9 nM at Stream 2), however, substantial N₂O accumulation was only observed at Stream 1 location. This finding suggests that the low Cu concentrations of ~ 6 nM were sufficient to enable the N₂O to N₂ conversion in Stream 2 sediments. The dissolved organic carbon concentration is lower at the Stream 2 location (2.1 mg C/L, Figure 5) which indicated that the fraction of dissolved Cu that is labile (i.e., not complexed with organic ligands) would be higher for Stream 2 (labile Cu in Stream 1: 0.55 nM and Stream 2: 4.8 nM). Thus, the presence of un-complexed Cu(II) at a concentration of ~ 4.8 nM enabled N₂O to N₂ conversion in Stream 2 controls.

N₂O was not detected in the headspace of Marsh 1 Cu-amended microcosms, however, we observed accumulation of N₂O in unamended control experiments in the initial days of incubation (max N₂O: 0.064 mmol-N/L). This observation suggests that the rate of N₂O to N₂ conversion was promoted by Cu amendment in Marsh 1 site (Figure 2s). The dissolved Cu concentration in the control samples was 48 nM, which is higher than the optimal range (3 to 10 nM) for N₂O transformation in pure culture studies (Granger and Ward, 2003; Glass and Orphan, 2012). The dissolved organic carbon (32 mg C/L) at the site is calculated to have decreased the bioavailability of Cu substantially (labile Cu in controls: 7.6 ± 5 nM) in the pH range studied, thus resulting in the transient N₂O accumulation in the headspace of the unamended controls.
4.2 Ammonium release during the incubations

In all the locations studied, a substantial amount of NH$_4^+$ was detected in the dissolved phase and remained constant throughout the experiment (Figure 2). Exchangeable NH$_4^+$ can be released from the solid phase to the fluid because of changes in the water-to-solid ratio, pH, and ionic strength. Alternatively, the NH$_4^+$ can result from the microbially-mediated dissimilatory nitration reduction to ammonium (DNRA) (Wang et al., 2008; Robertson and Thamdrup, 2017; Zhu et al., 2019; Liu et al., 2020; Wang et al., 2020). The ammonium was released into the fluid phase even before the onset of NO$_3^-$ reduction (Figure 2), and the concentration of NH$_4^+$ remained constant throughout the experiment. This observation indicated that most of the released NH$_4^+$ was due to exchange from the soils/sediments and not due to biological nitrate reduction. Additionally, the mass balance of extractable ammonium in soils/sediments indicates that the soils/sediments have the capacity to release the amounts of NH$_4^+$ observed in the fluid (Riparian 1: 0.05 mmol-N L$^{-1}$, Riparian 2: 0.14 mmol-N L$^{-1}$, Stream 1: 0.15 mmol-N L$^{-1}$, Stream 2: 0.14 mmol-N L$^{-1}$, and Marsh 1: 1.04 mmol-N L$^{-1}$).

4.3 Relationship between pH, dissolved metal content, and denitrification

During the initial 2-3 days of incubation, the pH increased from 5.0 to ~ 6.5 for Riparian 1 and Riparian 2 soils, from 7.6 to ~ 8.9 for Stream 1 and Stream 2 sediments, and from 7.0 to ~ 8.2 for Marsh 1 soils, and then remained relatively constant. The increase in pH values can be attributed to NO$_3^-$ and NO$_2^-$ reduction during denitrification. Previous study on riparian soils indicated that the pH increased from 5 to 7 and 5 to 9 in unbuffered NO$_3^-$ reduction experiments with low (111 µmol N g$^{-1}$) and high (500 µmol N g$^{-1}$) nitrogen loadings, respectively (Clement et al., 2005). In contrast, pH variation was limited (± 0.5) when denitrification occurred in carbonate-buffered lowland soils from Northern Italy (Castaldelli et al., 2019). Our previous study on the studied natural aquatic systems indicated that these soils and sediments lacked carbonate minerals (Yan et al., 2021); hence, the buffering capacity of the soils/sediments was likely inadequate to prevent the increase in pH upon NO$_3^-$ and NO$_2^-$ reduction.
The dissolved concentrations of Cu, Fe, and Mn were indirectly affected by nitrogen cycling in the incubation experiments. Due to a pH increase driven by denitrification, adsorption of metals to minerals phases increased and caused the dissolved concentrations of Cu, Fe, and Mn to decrease with time (Schultz and Grundl, 2004; Zhang et al., 2014). The decrease in Cu concentrations in Riparian 1 systems and controls and low-Cu amended sets of Marsh 1 soils (Figure 3a, 3d and 3m) likely resulted from an increase in Cu adsorption with increasing pH. We also observed that substantial amounts of Fe and Mn were released into the water in all of the incubation experiments after 24 h of incubation, indicating the reductive dissolution of Fe/Mn oxyhydroxides under anaerobic conditions (Zhang et al., 2014). The change in the ionic strength and pH of the soils/sediments during the preparation of the slurries could have also caused dispersion of colloidal Fe and Mn (Seta and Karathanasis, 1996). The released Fe decreased over time in the Riparian 1, Riparian 2, Stream 1, and Stream 2 sites. Under the conditions studied, dissolved Fe predominantly exists as Fe(II); the extent of Fe(II) sorption on clays, silica, and metal-oxide phases increases with an increase in pH (Schultz and Grundl, 2004; Nano and Strathmann, 2006; Zhu and Elzinga, 2014). Additionally, Fe(II) may serve as an electron donor for the abiotic reduction of NO$_3^-$ and NO$_2^-$ to form N$_2$ in soils and sediments (Eq 5-6) (Burgin and Hamilton, 2007; Klueglein et al., 2014; Robertson and Thamdrup, 2017; Liu et al., 2019; Otte et al., 2019; Rahman et al., 2019; Robinson et al., 2021).

\[
\text{NO}_3^- + 5\text{Fe}^{2+} + 12\text{H}_2\text{O} \rightarrow \frac{1}{2}\text{N}_2(\text{g}) + 5\text{Fe(OH)}_3 + 9\text{H}^+ \quad (5)
\]

\[
\text{NO}_2^- + 3\text{Fe}^{2+} + 7\text{H}_2\text{O} \rightarrow \frac{1}{2}\text{N}_2(\text{g}) + 3\text{Fe(OH)}_3 + 5\text{H}^+ \quad (6)
\]

Although the abiotic reduction of NO$_3^-$ to N$_2$ in the presence of Fe(II) is thermodynamically feasible, studies indicate that NO$_3^-$ can only be directly reduced by Fe(II) in the presence of mixed-valence Fe-bearing solids (green-rusts), or a catalyst, such as Cu(II), Sn(II), and Ag(I) (Moraghan and Buresh, 1977; Ottley et al., 1997; Davidson et al., 2003). Fe(II) adsorbed to iron-oxide surfaces is a stronger reducing agent than Fe(II) in dissolved form (Stumm and Sulzberger, 1992; Davidson et al., 2003), hence adsorbed Fe(II) might promote NO$_3^-$ reduction in the studied systems. Additionally, biological oxidation of structural Fe(II) in clay minerals, such as illite and nontronite, can be coupled with reduction of NO$_3^-$ to N$_2$ (Zhao et
al., 2013; Zhao et al., 2017). In our incubation studies, the dissolved Fe concentration decreased until 27, 18, and 6 days in Riparian 1, Riparian 2, and Stream 1 systems, respectively and then remained almost constant through the end of the experiments. Decreases in Fe concentration were only observed until the NO$_3$/$\text{NO}_2^-$ were completely consumed in these systems indicating that Fe(II) oxidation to Fe(III) oxides/hydroxides is coupled abiotically or biotically to NO$_3$/$\text{NO}_2^-$ reduction at Riparian 1, Riparian 2, and Stream 1 sites. Mass balance calculations indicated that NO$_3^-$ concentrations were sufficient to allow for consumption of Fe(II) in Riparian 1, Riparian 2, and Stream 1 systems to react with Fe(II) (Section S3 in SM). The values of $k_{ab}$ relative to $K_{\text{NO}_2^-}$ were high in Riparian 1, Riparian 2, and Stream 1 systems, suggesting that the reaction involving abiotic NO$_2^-$ reduction is substantial in these systems. On the other hand, the decrease in the Fe(II) concentration in Stream 2 experiments aligned with the pH increase during the initial 2-3 days of incubation, so the decrease in Fe concentration could also be due to increased adsorption at higher pH.

The addition of Cu affected Mn concentrations in Stream 2 experiments; the release of Mn was greater in sets amended with high Cu (Figure 3i). In stream 2 incubation experiments with high Cu loading, 250 µM Cu was added and only 0.60 µM remained in the dissolved phase after 24 h equilibration; the competitive adsorption of Cu(II) onto active sites of mineral-phases could have mobilized Mn(II) (~ 40 µM in this case) to the water. Prior studies have also observed the release of Mn(II) in the presence of Cu due to competitive adsorption (Kurdi and Donner, 1983; Traina and Doner, 1985). In Stream 1 and Marsh 1 studies, the concentration of dissolved Mn decreased with time (Figure 3i and 3o). Mn adsorption has been found to increase with pH on clay minerals, iron oxides/hydroxides and aluminum oxides. In reducing NO$_3^-$, Mn$^{2+}$ can also serve as an electron donor (Eq 7) to autotrophic denitrifiers belonging to the genera 

\[
\text{Mn}^{2+} + 0.4\text{NO}_3^- + 0.8\text{H}_2\text{O} \rightarrow \text{MnO}_2 + 0.2\text{N}_2 + 1.6\text{H}^+ \quad (7)
\]

Thus, the decrease in the concentration of Mn observed in Stream 1 and Marsh 1 samples can result from increased adsorption caused by a shift in pH or from Mn consumption by autotrophic denitrifiers.
4.4 Comparison of nitrogen cycling with materials from different systems

The studied aquatic systems, even different locations of the same site, showed varied trends in the reduction of nitrogen species in the incubation experiments. The total dissolved Cu concentrations in both locations of EFPC stream sediments (Stream 1 and Stream 2) were very low (3-10 nM), but transient N$_2$O accumulation only occurred in their unamended controls, whereas TB riparian wetland soils (Riparian 1 and Riparian 2) showed substantial N$_2$O accumulation despite having much higher dissolved background Cu concentrations of 30-50 nM. This disparity suggests that the speciation of Cu, and hence its bioavailability, plays a more important role than the total Cu content in controlling N$_2$O to N$_2$ conversion in the studied environmental systems.

Even after Cu addition, the accumulated concentrations of N$_2$O in the riparian wetland samples were higher than in the other locations. One possible explanation is that the riparian wetland incubation experiments were conducted at pH 5, whereas the incubations for marsh wetland soils (Marsh 1) and stream sediments (Stream 1 and Stream 2) were performed at neutral pH conditions. Acidic soils decrease the activity of the nitrous oxide reductase enzyme, leading to N$_2$O accumulation (Knowles, 1982; Simek and Cooper, 2002; Pan et al., 2012; Carreira et al., 2020). Optimal N$_2$O reduction has been observed in the pH range of 7.5-8.0; previous studies have observed substantial N$_2$O accumulation in the pH range of 6.0-6.5 (Pan et al., 2012; Carreira et al., 2020). Although the pH in the riparian wetland soils increased to ~ 6.5 in the first two days of incubation, i.e., before the onset of N$_2$O accumulation, it was in the range where a decrease in the activity of nitrous reductase enzyme has been observed (Pan et al., 2012; Carreira et al., 2020). Thus, Riparian 1 and Riparian 2 wetland soils could be a significant source of N$_2$O, not only because the bioavailable Cu is limited but also because they are acidic.

5. Geochemical significance and implications

Most pristine natural aquatic systems contain low solid-phase Cu, and hence they may have low availability of Cu for microbial denitrification. The limited set of studies on natural aquatic systems containing Cu at concentrations less than or equal to crustal abundances (441±63 nmol g$^{-1}$) support our
major finding that increased Cu concentrations can increase the extent of conversion of N$_2$O to N$_2$. At 26 µM dissolved Cu, Giannopoulos et al. (2020) concluded that greater availability of Cu led to less N$_2$O accumulation and higher abundance of Cu-dependent enzymes in wetland soils. A study on agricultural soils indicated that the application of organic fertilizer modified with 130 mM CuSO$_4$ decreased N$_2$O emissions substantially (Shen et al., 2020). However, these above-mentioned studies evaluated Cu concentrations that are relatively higher than the optimum range (3-10 nM) required for N$_2$O to N$_2$ transformation in pure culture studies. The concentrations of dissolved Cu in natural environments are typically low (< 200 nM), and the presence of inorganic/organic ligands can further decrease the bioavailability of Cu causing incomplete denitrification with N$_2$O accumulation. Our results indicated that without Cu amendment, substantial N$_2$O accumulation can take place in soils and sediments.

The selected sites represent different aquatic systems in geologically-distinct regions and contain low solid-phase and dissolved Cu (solid-phase: 45-280 nmol g$^{-1}$ and dissolved: 3-48 nM). Despite the differences in mineralogy, elemental composition, and aqueous-phase characteristics, the presence of dissolved Cu at trace levels (10-500 nM) decreased N$_2$O accumulation in all the sites. The response of riparian wetland soils (Riparian 1 and Riparian 2) to Cu addition was less pronounced than that of other studied sites, which highlights that the systems with acidic conditions like Riparian 1 and 2 can be substantial contributors of N$_2$O emissions even in the abundance of Cu.

Our study provides greater insight into the importance of Cu speciation on cycling of nitrogen species in environmental systems. The effect of Cu on N$_2$O accumulation was more closely associated with estimated labile-Cu concentrations than with total dissolved Cu concentrations. Dissolved Cu in the porewater of soils and sediments was substantially lower than the total solid-phase associated concentration, and its lability is lowered by its interactions with dissolved organic matter (Bourgeault et al., 2013; Zhang et al., 2014).

For systems with Cu limitations of complete denitrification, our results indicate that the addition of minor amounts of Cu can increase the rate of N$_2$O conversion in natural aquatic systems. Natural soils have been recognized as an important source of N$_2$O to the atmosphere and are estimated to release up to 5.6 Tg
N$_2$O-N yr$^{-1}$ (Tian et al., 2020). Current ecosystem models, such as DLEM, incorporate multiple environmental factors, including moisture content, temperature, nitrate and dissolved organic carbon concentration, and pH, in the estimation of N$_2$O emissions from terrestrial systems (Tian et al., 2015). These models do not account for the effect of trace metal micronutrient availability (Cu, Ni, Zn, Co, and Mo) on biogeochemical processes responsible for the release of greenhouse gas emissions. The inclusion of Cu as an additional parameter can help improve the accuracy of existing ecosystem models to predict N$_2$O emissions from soils and sediments. Addition of Cu at micronutrient levels to natural aquatic systems could potentially decrease N$_2$O release to the atmosphere. Addition of CuSO$_4$ in lakes is commonly practiced to inhibit algal growth. Previous studies indicate that Cu concentrations above 100 nM can decrease algal and bacterial populations in surface waters (Flemming and Trevors, 1989). Hence, addition of Cu at micronutrient levels (10-30 nM) is not expected to be toxic to aquatic organisms. Changes in Cu speciation in wetlands and stream sediments associated with hydrologic variation could also influence net N$_2$O emissions.

6. Conclusions

Through a combination of incubation experiments and a kinetic model we determined the effect of dissolved Cu at trace levels (10-500 nM) on the rate of N$_2$O reduction. Only the systems containing estimated labile Cu < 10 nM had substantial N$_2$O accumulation. Even with a small increase in dissolved Cu concentration, as observed in low-Cu-loaded incubation experiments, the rate of N$_2$O to N$_2$ conversion was significantly enhanced. The contribution of the abiotic reduction of NO$_2^-$ to N$_2$ by Fe(II) was significant at Riparian 1, Riparian 2, and Stream 1 locations. Riparian wetland soils showed higher N$_2$O accumulation than the other sites studied, indicating that the acidic pH conditions can enhance N$_2$O emissions from natural environments. The sites containing high concentrations of DOC (Riparian 1, Riparian 2, and Marsh 1) had less concentrations of dissolved Cu that were labile and showed greater N$_2$O accumulation. Our results indicate that including Cu bioavailability in ecosystem models could improve the accuracy of estimates of N$_2$O emissions from natural landscapes.
Acknowledgements

This project was supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, Subsurface Biogeochemical Research program through award no. DE-SC0019422 to Washington University. We acknowledge our collaborators, Pamela Weisenhorn, Edward J. O’Loughlin, and Kenneth M. Kemner at Argonne National Laboratory, Grace E. Schwartz and Scott C. Brooks at Oak Ridge National Laboratory, and Daniel I. Kaplan at Savannah River National Laboratory, who helped us in collecting samples from the selected aquatic systems. We thank Jinshu Yan, a doctoral student in the Department of Earth and Planetary Sciences for characterizing the soils/sediments used for the incubation experiments. ICP-MS measurements were performed in the Nano Research Facility (NRF) at Washington University in St. Louis. We also thank the McDonnell International Scholars Academy for the fellowship that is supporting Neha Sharma in her graduate program. We also thank James Ballard for assisting us in improving the quality of writing in this manuscript.

Appendix A. Supplementary Material

The supplementary material includes information on the recipe of the simulated water used for Cu uptake and incubation experiments, concentrations of solid-phase associated metals and nutrients, methodology and parameters used for estimating Cu speciation in the presence of dissolved organic carbon, the concentration of estimated labile Cu using NICA-Donnan model, and the calculations showing organic matter and Fe(II) requirements for nitrate reduction during the incubation experiments.

Research Data

Data associated with this article can be accessed at https://data.mendeley.com//datasets/t359pdcxxy/1.
References


Mehlhorn J., Besold J., Lezama Pacheco J. S., Gustafsson J. P., Kretzschmar R. and Planer-Friedrich B.


Shampine L. F., Gladwell I. and Thompson S. (2003) Solving ODEs with MATLAB,


Figures and Tables

Table 1: Cu loadings used for conducting incubation experiments, and dissolved Cu concentrations in the fluid during incubation experiments

<table>
<thead>
<tr>
<th>Site</th>
<th>Control</th>
<th>Low loading</th>
<th>High loading</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu added (µmol/g)</td>
<td>Dissolved conc. (nM)</td>
<td>Cu added (µmol/g)</td>
</tr>
<tr>
<td>Riparian 1</td>
<td>N.A</td>
<td>29±10</td>
<td>0.25</td>
</tr>
<tr>
<td>Riparian 2</td>
<td>N.A</td>
<td>41±9</td>
<td>0.25</td>
</tr>
<tr>
<td>Stream 1</td>
<td>N.A</td>
<td>3±1</td>
<td>0.25</td>
</tr>
<tr>
<td>Stream 2</td>
<td>N.A</td>
<td>6±2</td>
<td>0.50</td>
</tr>
<tr>
<td>Marsh 1</td>
<td>N.A</td>
<td>48±5</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Figure 1: Experimentally determined values of Cu uptake by wetland soils and stream sediments, for use in determining Cu loading in microcosm experiments. Here, Riparian 2 and Riparian 1 represent selected locations from the riparian wetland soil, Marsh 1 from marsh wetland soil, and Stream 2 and Stream 1 are locations from a stream sediment site.
Figure 2: Variation in the concentrations of different N- species (mmol-N L$^{-1}$) during the incubation experiments for (a-d) Riparian 1, (e-h) Riparian 2, (i-l) Stream 1, (m-p) Stream 2 and (q-t) Marsh 1. In case of low Cu loading, 0.25 µmol/g Cu was added in incubation experiments for Riparian 1, Riparian 2, and Stream 1 samples, 0.50 µmol/g for Stream 2 and 0.13 µmol/g for Marsh 1 incubations. High Cu loading amendments for incubation experiments were 5.0 µmol/g for Riparian 1 and Stream 1, 1.3 µmol/g for Riparian 2, 5.0 µmol/g for Stream 2, and 0.63 µmol/g for Marsh 1.
Figure 3: Variation in the concentrations of Cu, Fe and Mn during the incubation experiments for (a-c) Riparian 2, (d-f) Riparian 1, (g-i) Stream 1, (j-l) Stream 2 and (m-o) Marsh 1. In case of low Cu loading, 0.25 µmol/g Cu was added in incubation experiments for Riparian 1, Riparian 2, and Stream 1 samples, 0.50 µmol/g for Stream 2 and 0.13 µmol/g for Marsh 1 incubations. High Cu loading amendments for incubation experiments were 5.0 µmol/g for Riparian 1 and Stream 1, 1.3 µmol/g for Riparian 2, 5.0 µmol/g for Stream 2, and 0.63 µmol/g for Marsh 1.
Table 2: The values of Michaelis-Menten parameters of different reactions involved in carrying out denitrification at different sites as well as the pseudo first-order rate constant for abiotic reduction of NO$_2^-$ to N$_2$.

<table>
<thead>
<tr>
<th>Site</th>
<th>Condition</th>
<th>$V_{\text{max}}$ (mmol L$^{-1}$ day$^{-1}$)</th>
<th>$K_{\text{NO}_3^-}$ (mmol L$^{-1}$)</th>
<th>$k_{\text{ab}}$ (day$^{-1}$)</th>
<th>$K_{\text{NO}_2^-}$ (mmol L$^{-1}$)</th>
<th>$K_{\text{N}_2\text{O}}$ (mmol L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riparian 1</td>
<td>Control</td>
<td>0.41±0.02</td>
<td>12±0.5</td>
<td>99±2</td>
<td>0.072±0.004</td>
<td>6900±4</td>
</tr>
<tr>
<td></td>
<td>Low loading</td>
<td>0.41±0.05</td>
<td>11±0.7</td>
<td>99±4</td>
<td>0.072±0.006</td>
<td>24±0.2</td>
</tr>
<tr>
<td></td>
<td>High loading</td>
<td>0.41±0.03</td>
<td>15±0.3</td>
<td>98±5</td>
<td>0.068±0.002</td>
<td>3.5±0.4</td>
</tr>
<tr>
<td>Riparian 2</td>
<td>Control</td>
<td>0.25±0.08</td>
<td>1.1±0.1</td>
<td>2.2±0.3</td>
<td>0.68±0.08</td>
<td>11000±8</td>
</tr>
<tr>
<td></td>
<td>Low loading</td>
<td>0.25±0.07</td>
<td>1.1±0.3</td>
<td>2.2±0.9</td>
<td>0.22±0.06</td>
<td>0.48±0.1</td>
</tr>
<tr>
<td></td>
<td>High loading</td>
<td>0.25±0.03</td>
<td>1.7±0.3</td>
<td>2.2±0.4</td>
<td>0.33±0.07</td>
<td>0.21±0.09</td>
</tr>
<tr>
<td>Stream 1</td>
<td>Control</td>
<td>0.39±0.06</td>
<td>0.39±0.09</td>
<td>0.69±0.1</td>
<td>9.1±2</td>
<td>3.7±1</td>
</tr>
<tr>
<td></td>
<td>Low loading</td>
<td>0.39±0.07</td>
<td>0.41±0.1</td>
<td>0.71±0.2</td>
<td>9.7±2</td>
<td>0.72±0.4</td>
</tr>
<tr>
<td></td>
<td>High loading</td>
<td>0.39±0.01</td>
<td>0.49±0.01</td>
<td>0.64±0.01</td>
<td>9.5±1</td>
<td>0.00078±0.0003</td>
</tr>
<tr>
<td>Stream 2</td>
<td>Control</td>
<td>0.37±0.2</td>
<td>39±3</td>
<td>0.015±0.002</td>
<td>0.079±0.001</td>
<td>0.0085±0.0009</td>
</tr>
<tr>
<td></td>
<td>Low loading</td>
<td>0.37±0.7</td>
<td>40±4</td>
<td>0.015±0.007</td>
<td>0.0067±0.002</td>
<td>0.0024±0.0004</td>
</tr>
<tr>
<td></td>
<td>High loading</td>
<td>0.37±0.02</td>
<td>71±7</td>
<td>0.015±0.001</td>
<td>0.0035±0.001</td>
<td>0.0032±0.0007</td>
</tr>
<tr>
<td>Marsh 1</td>
<td>Control</td>
<td>0.27±0.07</td>
<td>0.47±0.2</td>
<td>0.00024±0.0001</td>
<td>0.058±0.008</td>
<td>0.14±0.04</td>
</tr>
<tr>
<td></td>
<td>Low loading</td>
<td>0.27±0.09</td>
<td>0.55±0.2</td>
<td>0.00027±0.0001</td>
<td>0.067±0.002</td>
<td>0.037±0.02</td>
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<tr>
<td></td>
<td>High loading</td>
<td>0.27±0.04</td>
<td>0.38±0.2</td>
<td>0.00029±0.0001</td>
<td>0.047±0.009</td>
<td>0.018±0.01</td>
</tr>
</tbody>
</table>
Figure 4: Experimental data together with the output of the optimized kinetic model for the evolution of N-containing species during the incubation experiments using the parameters obtained from the kinetic model for (a-c) Riparian 1, (d-f) Riparian 2, (g-i) Stream 1, (j-l) Stream 2 and (m-o) Marsh 1.
Figure 5: Final dissolved organic carbon concentrations in the incubation experiments with soils and sediments of different natural aquatic systems.
Figure 6: Speciation of dissolved Cu at different concentrations for (a-c) Riparian 2 (d-f) Riparian 1 and (g-i) Stream 2 (j-l) Stream 2 and (m-o) Marsh 1. The concentrations of Cu selected for determining the speciation are based on the dissolved concentration of Cu in the incubation experiments (Table 1). Here, Cu-S1 shows Cu bound to carboxylic acids of organic carbon and Cu-S2 is the Cu bound to phenolic groups on organic carbon. Shaded areas indicate the pH range over the course of the experiment.
SUPPLEMENTARY MATERIAL FOR

Copper availability governs nitrous oxide accumulation in wetland soils and stream sediments

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Geochimica et Cosmochimica Acta

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## Supplementary material details

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<tr>
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<td>Characterization of soils and sediments collected from different aquatic systems</td>
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<td>6</td>
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<td>Section S3</td>
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<td>7</td>
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<tr>
<td>Figure S1</td>
<td>Variation in the concentration of NO$_3^-$ during incubation period at different sites studied. The scale on the plots is selected to show changes in in NO$_3^-$ for the earliest reaction times.</td>
<td>8</td>
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Table S1: Concentration of major elements and species in the simulated site water used for uptake studies and microcosm experiments

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ANL (nmol/g)</th>
<th>ORNL (nmol/g)</th>
<th>TB (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.0</td>
<td>7.6</td>
<td>5.0</td>
</tr>
<tr>
<td>Ionic Strength</td>
<td>1.7 mM</td>
<td>7.0 mM</td>
<td>0.30 mM</td>
</tr>
</tbody>
</table>

**Concentration (µM)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ANL (µM)</th>
<th>ORNL (µM)</th>
<th>TB (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>170</td>
<td>450</td>
<td>60</td>
</tr>
<tr>
<td>K⁺</td>
<td>170</td>
<td>71</td>
<td>5.3</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>370</td>
<td>1100</td>
<td>25</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>290</td>
<td>390</td>
<td>27</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>490</td>
<td>2700</td>
<td>160</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>580</td>
<td>260</td>
<td>7.6</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>8</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

Table S2: Characterization of soils and sediments collected from different aquatic systems (Yan et al.)

<table>
<thead>
<tr>
<th>Site</th>
<th>Cu (nmol/g)</th>
<th>Mn (nmol/g)</th>
<th>Fe (µmol/g)</th>
<th>C (%)</th>
<th>S (%)</th>
<th>NH₄⁺ (µmol/g)</th>
<th>NO₂⁻ (µmol/g)</th>
<th>NO₃⁻ (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riparian 1</td>
<td>48</td>
<td>220</td>
<td>47</td>
<td>1.3</td>
<td>0.02</td>
<td>1.1</td>
<td>BDL</td>
<td>0.14</td>
</tr>
<tr>
<td>Riparian 2</td>
<td>260</td>
<td>701</td>
<td>460</td>
<td>6.0</td>
<td>0.09</td>
<td>2.7</td>
<td>BDL</td>
<td>0.00</td>
</tr>
<tr>
<td>Stream 1</td>
<td>160</td>
<td>3200</td>
<td>204</td>
<td>3.0</td>
<td>0.10</td>
<td>2.7</td>
<td>BDL</td>
<td>0.14</td>
</tr>
<tr>
<td>Stream 2</td>
<td>98</td>
<td>5600</td>
<td>420</td>
<td>0.46</td>
<td>0.02</td>
<td>2.8</td>
<td>BDL</td>
<td>0.43</td>
</tr>
<tr>
<td>Marsh 1</td>
<td>280</td>
<td>1960</td>
<td>420</td>
<td>9.0</td>
<td>0.24</td>
<td>21</td>
<td>BDL</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Here, nutrients represent the extractable values from soils and sediments. Total Cu, Mn, and Fe concentrations present in soils and sediments were obtained using microwave-assisted digestion technique. Carbon and sulfur percent were estimated using CHNS analyzer. The detection limit of NO₂⁻ is 0.02 µmol/g.
Two different binding sites are considered in the NICA model, type 1 and type 2, corresponding to carboxylic (low affinity) and phenolic (high affinity) sites respectively (Benedetti et al., 1995).

\[
Q_i = \frac{n_{i,1} Q_{\text{max}1,H}}{\sum (k_{i,1} c_i)^{n_{i,1}}} + \frac{n_{i,2} Q_{\text{max}2,H}}{\sum (k_{i,2} c_i)^{n_{i,2}}}
\]

where, \(c_i\) (mol. L\(^{-1}\)) is the concentration of metal; \(Q_i\) is the amount of bound ion described by two identical binding expressions, one each for the carboxylic- (1) and phenolic-type (2) site distributions. \(Q_{\text{max}1,H}\) and \(Q_{\text{max}2,H}\) are the maximum proton binding capacity of humic substances within each distribution (mol kg\(^{-1}\)); \(p_1\) and \(p_2\) account for intrinsic heterogeneity of humic substances; \(k_{i,1}\) and \(k_{i,2}\) are median values for affinity distributions for ion, and \(n_{i,1}\) and \(n_{i,2}\) are used to describe the nonidealities of the ion-binding to each distribution. The ratios \(\frac{n_{i,j}}{n_{H,j}}\) with \(j = 1\) or 2 reflect the average stoichiometry of ion binding.

The charge on humic substances is neutralized by the nonspecific binding of counter-ions and exclusion of co-ions within the Donnan volume, \(V_D\) (L·kg\(^{-1}\)), as described by the empirical relationship.

\[
\log V_D = b (1 - \log I) - 1
\]

Here, \(I\) is ionic strength and \(b\) is an empirical parameter describing the variation of Donnan volume with ionic strength (Benedetti et al., 1996). The values of parameters used in estimating Cu speciation are listed in Table S3.

Humic substances are normally assumed to be the main binding substances (Ren et al., 2015), and their concentrations were determined from the dissolved organic carbon (DOC) concentrations. Humic substances account for 60% of DOC in natural water systems (Zhang and Davison, 2000; Gueguen et al., 2011), and they comprise 50% carbon, so concentration of HS was assumed to be 1.2 times the DOC concentration. The pH, temperature, total dissolved elements (Na, Mg, K, Ca, Cl, NO\(_3\), SO\(_4\), and PO\(_4\)) (Table EA1), and dissolved Cu, Fe, and Mn were used as the input parameters for determining Cu speciation.
Table S3: Parameters used in NICA-Donnan model for determining Cu speciation in the presence of DOC (Xu et al., 2016)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>0.57</td>
</tr>
<tr>
<td>$Q_{\text{max},1,H}$</td>
<td>5.88</td>
</tr>
<tr>
<td>$p_1$</td>
<td>0.59</td>
</tr>
<tr>
<td>$log\hat{k}_{H,1}$</td>
<td>2.34</td>
</tr>
<tr>
<td>$n_{H,1}$</td>
<td>0.66</td>
</tr>
<tr>
<td>$log\hat{k}_{\text{Cu},1}$</td>
<td>0.26</td>
</tr>
<tr>
<td>$n_{\text{Cu},1}$</td>
<td>0.53</td>
</tr>
<tr>
<td>$Q_{\text{max},2,H}$</td>
<td>1.86</td>
</tr>
<tr>
<td>$p_2$</td>
<td>0.70</td>
</tr>
<tr>
<td>$log\hat{k}_{H,2}$</td>
<td>8.60</td>
</tr>
<tr>
<td>$n_{H,2}$</td>
<td>0.76</td>
</tr>
<tr>
<td>$log\hat{k}_{\text{Cu},2}$</td>
<td>8.26</td>
</tr>
<tr>
<td>$n_{\text{Cu},2}$</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Table S4: Estimated labile concentrations of Cu in the microcosms using NICA-Donnan model

<table>
<thead>
<tr>
<th>Site</th>
<th>Estimated labile concentration (nM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Riparian 1</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>Riparian 2</td>
<td>1.4 ± 0.8</td>
</tr>
<tr>
<td>Stream 1</td>
<td>0.55 ± 0.3</td>
</tr>
<tr>
<td>Stream 2</td>
<td>4.8 ± 0.9</td>
</tr>
<tr>
<td>Marsh 1</td>
<td>7.6 ± 5</td>
</tr>
</tbody>
</table>

*Labile Cu concentration is the sum of Cu, CuOH$^+$, and Cu(OH)$_2$. The values represent the average of the labile concentrations between the pH range studied. pH varied between 5-6 in wetland soils; 7.6-8.6 in stream sediments, and 7-8 in marsh wetland soils.
Section S2: Estimation of organic carbon requirements for complete reduction of nitrate

\[
\text{CH}_2\text{O} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 4\text{e}^- + 4\text{H}^+ \quad \text{Eq S3}
\]

\[
\text{NO}_3^- + 5\text{e}^- + 6\text{H}^+ \rightarrow \frac{1}{2} \text{N}_2 + 3\text{H}_2\text{O} \quad \text{Eq S4}
\]

\[
5\text{CH}_2\text{O} + 4\text{H}^+ + 4\text{NO}_3^- \rightarrow 5\text{CO}_2 + 2\text{N}_2 + 7\text{H}_2\text{O} \quad \text{Eq S5}
\]

Total nitrate available = 0.05 mmol

Total organic carbon (OC) requirement = 0.0625 mmol

| Site       | Organic Carbon (%) | Total organic carbon (mmol) | \(
\frac{\text{TOC}_{\text{total}}}{\text{OC}_{\text{required}}}
\) |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Riparian 2</td>
<td>6.0</td>
<td>13</td>
<td>208</td>
</tr>
<tr>
<td>Riparian 1</td>
<td>1.3</td>
<td>2.7</td>
<td>43</td>
</tr>
<tr>
<td>Stream 1</td>
<td>3.1</td>
<td>6.3</td>
<td>101</td>
</tr>
<tr>
<td>Stream 2</td>
<td>0.46</td>
<td>0.77</td>
<td>12</td>
</tr>
<tr>
<td>Marsh 1</td>
<td>9.0</td>
<td>19</td>
<td>304</td>
</tr>
</tbody>
</table>

*Here, total organic carbon represents the total amount of organic carbon present in the solid phase (2.5 g of soil/sediment) used for incubation experiments.
Section S3: Estimation of nitrate requirements for abiotic reaction with Fe(II)

\[ \text{NO}_3^- + 5\text{Fe}^{2+} + 12\text{H}_2\text{O} \rightarrow \frac{1}{2}\text{N}_2(\text{g}) + 5\text{Fe(OH)}_3^- + 9\text{H}^+ \]

Riparian 1:
Decrease in Fe: 7.1 µmol
NO$_3^-$ required: 1.4 µmol

Riparian 2:
Decrease in Fe concentration: 72 µmol
NO$_3^-$ required: 14 µmol

Stream 1:
Decrease in Fe concentration: 0.084 µmol
NO$_3^-$ required: 0.017 µmol
Figure S1: Variation in the concentration of NO$_3^-$ during incubation period at different sites studied. The scale on the plots is selected to show changes in NO$_3^-$ for the earliest reaction times.
References


