

Micronutrient Management of Nitrous Oxide Emissions

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Abstract: Agriculture is the primary anthropogenic source of nitrous oxide (N₂O), a potent greenhouse gas, and mitigating soil emissions without compromising food security is critical for climate targets. We assess the role of copper (Cu), as a micronutrient, in regulating nitrogen-cycling enzymes. Using mesocosm experiments and long-term field trials, we demonstrate that Cu addition, coupled with pH modification, significantly reduces N₂O fluxes. Metagenomic and metatranscriptomic analyses reveal that Cu availability regulates the expression of *moxA* (a Cu-containing enzyme) and its coupling with nitrous oxide reductase (*nosZ*). These findings suggest that targeted Cu amendments—via direct application or silicate-enhanced weathering—offer a scalable strategy to decrease agricultural N₂O emissions while maintaining soil productivity.

Main Text: Achieving key climate goals will require rapid and aggressive reductions in all greenhouse gas (GHG) emissions (1). However, integrated assessment modeling suggests that there will be significant residual or difficult-to-abate emissions at net-zero, a considerable fraction of which (>35%, (2)) will be represented by nitrous oxide (N₂O) – a long lived and potent GHG that currently contributes to 6.4% of global GHG emissions (3). The majority (51%) of anthropogenic N₂O emissions are linked to the application of reactive nitrogen (N)-fertilizers and manure, and these emissions are projected to further increase to meet food demands for a growing population (4, 5). Soil N-cycling is controlled primarily by plant and microbial uptake and assimilation and microbial transformations. Microbial transformations include the oxidation and reduction reactions within which N₂O is created as an intermediate product (4, 6, 7). Attempts to decrease N₂O emissions have largely focused on better nutrient management (8) although efforts to alter microbial N-cycling have become increasingly common (e.g., nitrification inhibitors (9–12)).

A sound understanding of the enzymes shaping soil N₂O fluxes provide a foundation to explore additional N₂O remediation pathways. For example, the enzyme nitrous oxide reductase (*nosZ*) facilitates the last step of denitrification by catalyzing the two-electron reduction of N₂O to atmospheric nitrogen (N₂) (13). *nosZ* activity has a parabolic relationship with pH, with highest activity at circumneutral pH values (14). As such, increasing soil pH has the potential to reduce N₂O emissions across a range of managed systems (15–18). The activity of manganese oxidase (*moxA*), which oxidizes soluble Mn(II) into MnO₂ (19), can also play a role in decreasing N₂O emissions through the Mn-coupled denitrification or Mn-mediated oxidation of ammonium to atmospheric dinitrogen (20–22).

Copper (Cu) is an important metal cofactor for both *nosZ* and *moxA* (13, 19). Copper limitation resulting in increased N₂O fluxes has been observed in denitrifying bacterial cultures, soybean root nodules, wetland soils, bare soils, and cabbage (23–28). However, no study to date has explored the impacts of Cu on N₂O over a range of agricultural crop soils at varied pH values, its impacts on Mn-cycling, and the potential coupling of Mn-N-cycling through *moxA* and *nosZ*, which both encode Cu-dependent enzymes. Similarly, studies examining the impacts of Cu on maize, which accounts for the majority of agricultural N₂O emissions in the US (29), are lacking.

Here we present results from mesocosm experiments and a long-term field trial of Cu addition to agricultural soils. We demonstrate that Cu can curb N₂O fluxes from maize at the mesocosm and field scale. We build on these results to demonstrate that this process could significantly reduce total U.S. agricultural N₂O fluxes, with the potential to significantly lower U.S. residual GHG emissions without decreasing fertilizer use. These findings reveal the necessity of understanding the potential for Cu additions as a scalable, risk-assessed strategy for agricultural N₂O emission control.

Response of N₂O fluxes to changes in Cu and pH in mesocosms and a long-term field trial:

We continuously monitored N₂O fluxes from large (121 L) mesocosms constructed from field-sampled agricultural soil planted with maize in a controlled greenhouse setting (fig. S1). We adjusted levels of soil Cu and pH through Cu addition (see Methods, table S1, data S1) and addition of limestone soil amendments. We allowed the soil to equilibrate after Cu priming (see Supplementary text, fig. S2). In all iterations of the experiment (Runs 1-6), Cu-amended mesocosms had, on average, lower N₂O emissions than in the control mesocosms (table S2, data S2), and we observed statistically significant reductions in N₂O between the experimental and

control mesocosms when the pH was neutral to high (Runs 1 and 6, table S2). We found that at higher soil pH, when denitrification is more efficient, Cu additions further decrease N₂O emissions, whereas under acidic conditions, the mitigation potential of Cu is more muted (Fig. 1, fig. S3).

Because the N₂O fluxes (data S2) were measured over a range of Cu concentrations (21-132 ppm solid phase, 0.2-10.9 ppm exchangeable fraction; data S1) driven by a range of application rates (1.7-21.0 kg Cu/ha; table S1) and pH values (5.15-7.30; data S1), we also explored the statistical significance through a multivariate nonlinear regression of the N₂O fluxes, pH, and solid phase Cu concentration (see Equation 1 in Methods). Results suggest there are interacting effects of Cu and pH on N₂O emissions, with the lowest N₂O emissions occurring at higher Cu values and higher pH (fig. S3); for example, when comparing at treated mesocosms from all Runs with a pH above 6.5 and a solid phase Cu concentration of above 33.67 ppm to control mesocosms, N₂O emissions were $63.6 \pm 20.1\%$ ($\pm 95\%$ CI) lower on average (Fig. 1A).

We also used a random forest machine learning framework, which predicts that Cu is the most dominant factor shaping N₂O fluxes (fig. S4). In alignment with existing theory, random forest analysis also predicts that surface soil moisture and soil pH are the next two most important features for predicting N₂O fluxes in our system. We found no statistically significant changes in total plant biomass (data S1) between treatments, indicating varying productivity was unlikely to be a key variable.

We evaluate whether the Cu-N₂O linkage reported from mesocosm experiments translates to the field scale by exploring N₂O fluxes from a long-term field trial at the University of Illinois Energy Farm. In this trial, addition of crushed silicate rock resulted in Cu addition of 3.6 kg/ha over 4 years (30–32). Soil pH increased from 6.1 to 6.9 as basalt feedstock weathered (32), along with increases in both soil exchangeable (from 43 to 62 ppb (32)) and solid phase Cu (from 18.6 to 21.6 ppm (table S3)) as a response to crushed silicate rock amendment (see methods for a description of Cu analytical procedures). The N₂O fluxes were 22% lower (0.0095 kg N/ha/d lower), on average, in treated, Cu enriched plots (cumulative emissions shown in Fig. 1B) (30, 31).

Metagenomic and metatranscriptomic insights into the mechanism of Cu impacts on soil N₂O flux:

We next employ soil metagenomic and metatranscriptomic analyses to understand the mechanisms driving the response of soil N₂O fluxes to pH and Cu concentrations. In the mesocosm soils, there is a clear signal that the microbiome began participating in more active Cu-cycling with elevated Cu concentration following amendment (Fig. 2A, fig. S5). Specifically, we observe strong positive correlations between the soil total Cu levels and the cumulative expression of (1) Cu transporter genes (Fig. 2A), (2) Cu sensing and regulation, and (3) Cu chelation genes and sequestration (fig. S5, data S3). These findings were replicated in field trials, where metagenomics analyses indicated that these 3 groups of Cu-responding genes are of significantly greater abundance in Cu-enriched rock grain mesh bags and basalt-treated bulk soils relative to untreated control bulk soils (fig. S5, data S3). Together, these findings indicate that the belowground microbiomes respond strongly to increases in Cu availability. The total soil concentrations of Cu did not affect either the amount of total DNA or the amount of total RNA extracted from soil mesocosms (fig. S6, data S3). These findings suggest that, at the levels applied, Cu does not exert anti-microbial or inhibitory effects on the bulk soil microbial community.

We report, for the first time, that the enhancement in Cu cycling following CuCl₂ and Cu enriched rock-amendments are linked to parallel increases in microbial manganese (Mn) cycling

(data S3)— including Mn-transport (Fig. 2B) and Mn-oxidation (Fig. 2C). At the heart of this crosstalk, the *moxA* encoding a multi-copper Mn-oxidase (Fig. 2C), may represent an important regulatory element that integrates the microbial response to these trace metals. Further, the increased *moxA* expression in response to Cu may limit the negative effects of high intracellular Cu levels as the *moxA* enzyme itself sequesters 4 atoms of Cu per molecule in its Cu-centers. Subsequently, the Cu-activated *moxA* can harness the increased uptake of Mn^{2+} and oxidize this extra Mn^{2+} into Mn-oxides, generating energy. Previously, it was demonstrated that coupling Mn-oxidation to denitrification steps in a process termed nitrate-dependent manganese oxidation (NDMO), which includes the reduction of all denitrification intermediates, is more thermodynamically favorable than denitrification alone (20, 22). We find that NDMO processes were enhanced in response to added Cu as the community expressing *moxA* became phylogenetically more similar, represented by shorter distances, to that expressing *nosZ* – the central gene enzyme catalyzing the reduction of N_2O to N_2 (Fig. 2D-F, data S3). The shortened phylogenetic distance between *moxA* and *nosZ*-expressing communities indicate a significant overlap in microbes that express the two genes simultaneously, supporting a central role of NDMO processes in N_2O to N_2 reduction in Cu-amended soil.

To provide further support for the central role of Cu-mediated Mn-oxidation in the microbial reduction of N_2O to N_2 , we performed global genomic analyses of >74,000 bacterial genomes published to date (see methods). Our analyses reveal that while *moxA* was only found in 5.6% of all bacterial genomes, its occurrence was ~2-fold significantly higher in *nosZ*-carrying genomes, being recorded in 10.1% of these (Fisher’s Exact Test, $P < 0.001$; fig. S7).

Further to the observed Cu-mediated effects, we analyzed how pH and Cu interact to directly affect the microbial conversion of N_2O to N_2 (carried out by *nosZ*) relative to the generation of N_2O from NO (carried out by *norBC*). The resulting ratio, *nosZ/norBC*, provides a more integrated view of possible N_2O fluxes as it considers N_2O reduction over N_2O production. We find that *nosZ/norBC* ratios were greatest under high pH/high Cu in the mesocosms and in rock mesh bags in the field trials representing microsites of localized high pH and high Cu (Fig. 2G, Fig. 2I, data S3). Similar trends are recorded in the expression and abundance of *moxA* (Fig. 2H, Fig. 2J, data S3). These results indicate that in addition to the major effects of soil pH, increases in soil Cu levels promote reduction of N_2O to N_2 , relative to N_2O generation through enhancement in the *moxA*-driven NDMO processes.

In summary, our multi-omics approach provides evidence in support of the major reported effect of pH on regulating N_2O emissions but also presents data on the previously unrealized role of Cu and Mn cycling in curbing N_2O evolution through their coupling to the N cycle.

The large-scale potential for Cu-based N_2O reduction in U.S. Croplands:

We next assess the potential for Cu amendments to curb N_2O emissions across the continental US. In this assessment, we use multivariable nonlinear regression of solid Cu, pH, and N_2O based on the mesocosm data to predict a percent change in annual national US N_2O fluxes. For this, we modeled simultaneous changes to soil pH and Cu concentration across all contiguous US land and cropland (Fig. 3 and fig. S8). We then scale this series of assumed soil pH and Cu treatment effects to national N_2O flux estimates (see Methods).

As shown in Fig. 3 and fig. S8, leaving soil pH unchanged while increasing bulk Cu on cropland areas to 30 ppm, 40 ppm, and 50 ppm—notably, all of which remain well below accepted

agricultural toxicity thresholds developed for use of Cu as an organic fungicide among other Cu application pathways (e.g., 33)—results in a predicted change in N₂O emissions of -12.88, -24.58, and -34.74%, respectively. Combining the same changes to soil Cu inventory with increases in soil pH to a target of 7, our estimated national percent change in N₂O emissions amounts to -21.12, -37.12, and -52.37% (for 30 ppm, 40 ppm, and 50 ppm values of assumed soil Cu, respectively). These reductions translate to between 4.8 billion and 20.6 billion USD saved annually using a social cost of carbon (SCC) of \$185/tonne CO₂e (circa ref. (34)). The magnitude of reductions is generally most favorable where N₂O emissions are the highest: in the US Midwest and along the Mississippi river floodplain (Fig. 3D, fig. S8).

Potential for Implementation:

We report significant reductions in soil N₂O fluxes in response to Cu additions in both a controlled environment mesocosm and enhanced weathering field settings. Continuous N₂O flux measurements show that the reduction in N₂O from Cu addition is not intermittent but represents a baseline shift and a reduction in the cumulative impact of high-flux intervals (often called hot moments). Further field trials exploring this relationship will be essential for accurately predicting changes in N₂O flux from soil Cu addition. Nonetheless, the significant shifts in soil N₂O production and the clear mechanistic basis for these shifts provided by metagenomic and metatranscriptomic analyses provide impetus to consider whether implementation of soil Cu amendment for GHG management could be feasible, especially considering that Cu as a soil amendment is already incorporated into US agriculture as a micronutrient and as a fungicide, and the most common direct Cu amendments are relatively inexpensive (e.g., \$4.40/kg CuSO₄, table S4). We expect that the relatively small amount of Cu required to induce significant differences in N₂O flux will help to bring down the carbon intensity and overall cost of Cu transport. An initial spatially explicit technoeconomic assessment suggests there are locations where the cost of mitigation could be less than \$50/tonneCO₂e for a 10-year integration period (Fig. 3F, fig. S9, see Supplementary Materials for Methods used to determine cost of deployment). Lastly, slow-release Cu addition from silicate enhanced weathering treatments would provide a free co-benefit for the carbon dioxide removal strategy (35).

Currently, N₂O comprises 6.4% of global GHG emissions, and ~30% of annual agriculture, forestry, and other land use emissions (2). Even under the strictest shared socioeconomic pathways scenario, currently predicted N₂O emission declines are modest given the need to increase fertilizer use to meet the agricultural demand for a growing population (1, 2). It is projected that we will need to double agricultural production by midcentury in order to feed a growing population (36) and losses in yield driven by climate change are already occurring (1, 37). These results may be optimistic given increasing evidence that localized increases in rain driven by climate change will likely increase nitrogen losses from fields in the Northeast and corn belt of the United States (e.g., 38, 39), which implies that more nitrogen will need to be added in order to maintain the same rate of plant uptake of nitrogen. Embracing this view of future nitrogen use, it is easy to make a case that there is a clear and urgent need to try to find solutions to N₂O emissions while still increasing food production. We suggest Cu additions to agricultural soils may offer a novel pathway for addressing current N₂O emissions and mitigating future residual emissions. Our results highlight the need for further research on regulatory controls of microbially driven soil N₂O emissions from agricultural systems, along with technoeconomic analysis of potential sources of Cu as a soil amendment.

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Supplementary Materials:

Materials and Methods

Supplementary Text

Figs. S1 to S10

Tables S1 to S6

References (40-70)

Data S1 to S3

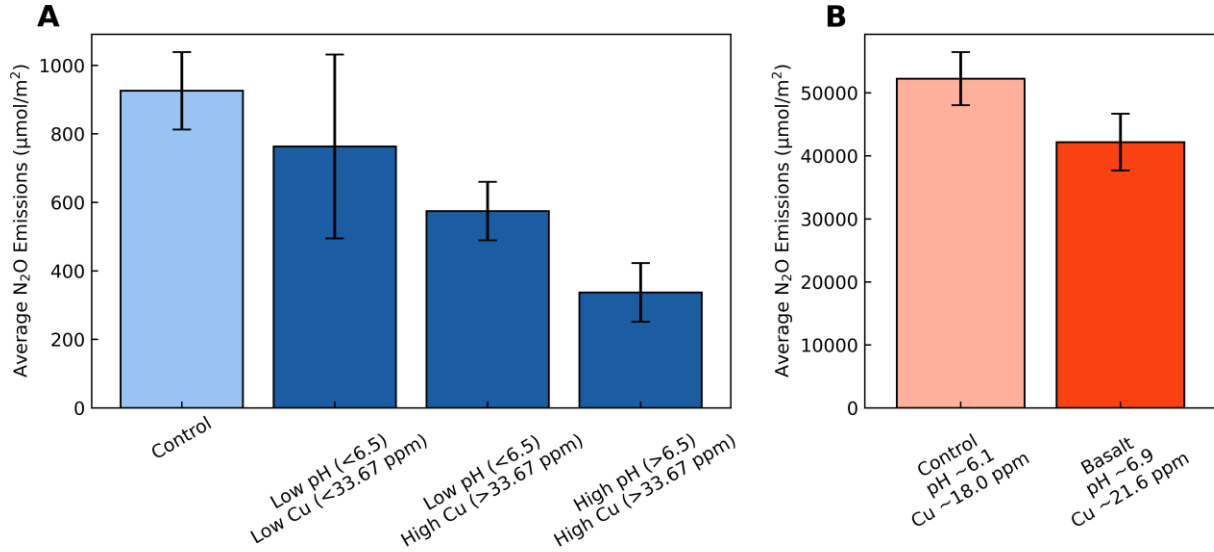
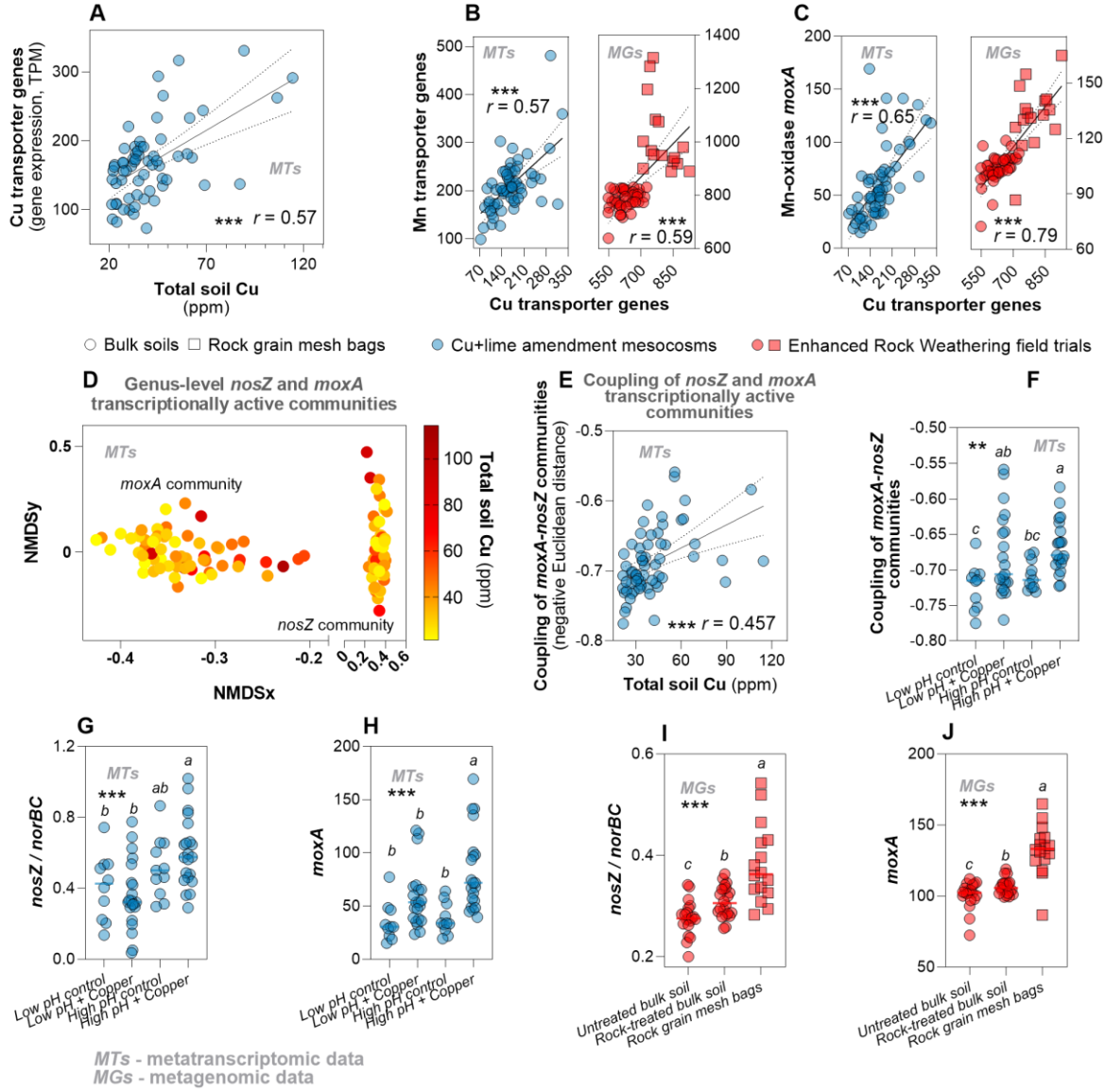


Fig. 1. Average cumulative N₂O emissions (μmol/m²) over the experimental periods for mesocosms and field trial. The average cumulative N₂O emissions (μmol/m²) for (A) mesocosms growing maize were calculated over the same experimental time period (24 days) for each run and are grouped by control mesocosm (light blue) and experimental mesocosm—copper and pH—categories (dark blue). Copper threshold was determined based on the 90th percentile of solid phase copper concentration from the control mesocosms. Error bars represent standard error. The average cumulative N₂O emissions (μmol/m²) for (B) field trial plots with a maize-soy rotation are integrated over each summer growing season and summed for the entire four-year dataset. The field trial emissions are grouped by control (pink) and basalt amended (red) categories. Error bars represent standard error calculated by summing mean flux variances in quadrature. [Cu] and pH values are indicated by averages from the final flux sampling year (2019). See supplemental materials for more experimental details.



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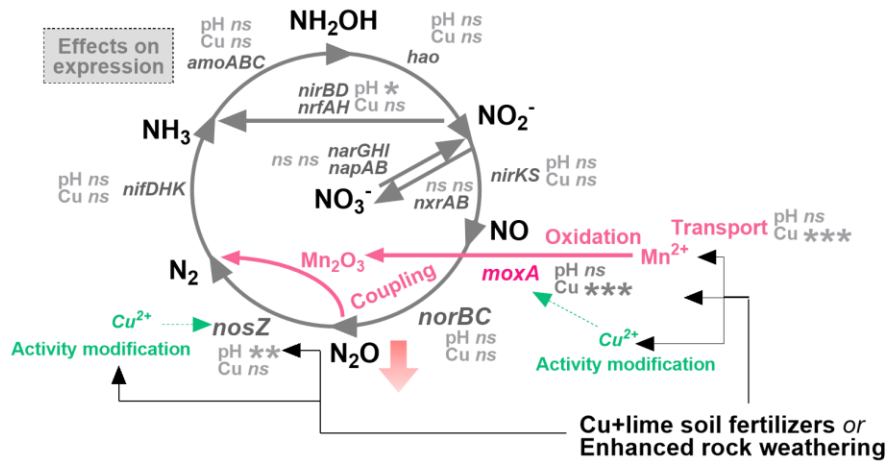


Fig. 2. Multi-omics analyses of the Cu-mediated reduction in soil N₂O emissions. (A) Cumulative expression of Cu transporter genes in the mesocosm soil microbial community in response to soil total Cu levels after Cu-amendment – strong positive correlation (Pearson's correlation test, $P < 0.001$); (B) Correlation between the cumulative expression of Cu and Mn transporter genes in mesocosms and enhanced rock weathering field trials ($P < 0.001$); (C) Correlation between the cumulative expression of Cu transporters and that of manganese oxidase *moxA* gene in soil microbial communities of mesocosms (blue; $P < 0.001$) and enhanced rock weathering field trials (red, $P < 0.001$); (D) Non-metric multidimensional scaling (NMDS) analysis of the *nosZ*-expressing and *moxA*-expressing microorganisms using genus-level taxonomy in mesocosm experiments – the two communities exhibit shortened phylogenetic distance, more organisms potentially expressing both genes, as the concentration of soil total Cu increases (color-coded yellow to red); (E) Coupling between *nosZ-moxA* communities correlates positively with soil total Cu concentrations ($P < 0.01$). Coupling was measured as the negative Euclidean distance between each *moxA* community against the average *nosZ* community (spacing was based on Jaccard dissimilarity index) in mesocosms; (F) The level of *nosZ-moxA* coupling according to the mixed pH- and Cu-groups in mesocosms (ANOVA, $P < 0.01$ with Post-Hoc FDR multiple-comparison corrections); (G) *nosZ/norBC* ratio according to the mixed pH/Cu grouping in mesocosms; (H) *moxA* expression according to the mixed pH/Cu grouping in mesocosms; (I) The *nosZ/norBC* ratio according to substrate at the enhanced rock weathering field trials; (J) The *moxA* gene abundance according to substrate at the enhanced rock weathering field trials; (K) Holistic N-cycle analyses reveal multiple points of Cu and pH-coupled and independent control on N₂O. Coupling of the *moxA*-catalyzed Mn-oxidation to the *nosZ*-catalysed N₂O reduction to N₂ may make the latter process more thermodynamically favorable. Asterisks of ***, **, and * represent p-values of < 0.001 , < 0.01 , and < 0.05 respectively.

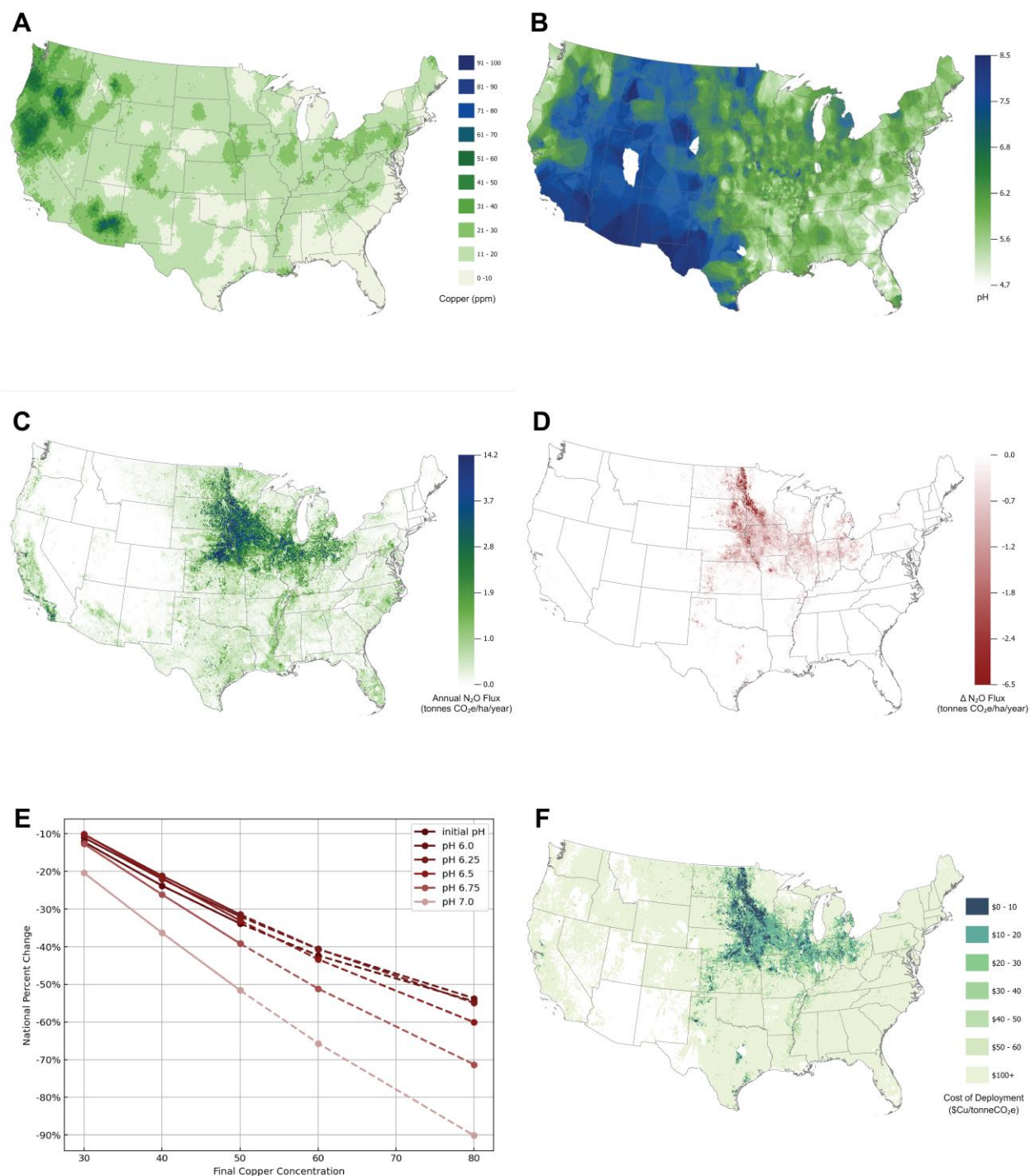


Fig. 3. Maps of Cu, pH, and N₂O fluxes, and changes in N₂O given Cu amendments in the United States. (A) Bulk Cu concentration, (B) soil pH, (C) annual total N₂O emissions from (29), (D) tonnes of N₂O (in CO₂e) saved annually by Cu amendments on croplands to achieve a Cu concentration of 50 ppm, and (E) national percent change in N₂O emissions annually given increases in soil Cu concentration to 30, 40, 50, 60, 80, and 100 ppm bulk Cu and increases in soil pH to none, 6.0, 6.25, 6.5, 6.75, and 7.0 when possible and determined beneficial based on the regression (Equation 1). Prediction lines are muted past 50 ppm, at which point different nations

have different Cu threshold recommendations. (F) Modeled cost of the N₂O emissions reduction strategy in USD spent on CuSO₄ per tonne CO₂e avoided by elevating bulk soil Cu concentration to 50 ppm.

Supplementary Materials for

Micronutrient Management of Nitrous Oxide Emissions

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The PDF file includes:

Materials and Methods
Supplementary Text
Figs. S1 to S10
Tables S1 to S6
References (40-70)

Other Supplementary Materials for this manuscript include the following:

Data S1 to S3

Materials and Methods

Overview

We assessed the impact of various levels of copper on N₂O fluxes within maize (*zea mays*, Reid's Yellow Dent Open Pollinated Corn Seed, Bradley Seed Brand) mesocosms in a Yale Science Building research greenhouse (affiliated with Yale University's Marsh Botanical Garden's plant growth facility). We established average growing season conditions with the Argus Prime automation system. We measured greenhouse gas fluxes, soil pH, topsoil Sikora buffer pH, temperature, soil moisture, and copper concentration (solid phase and exchangeable fraction). We performed 6 iterations of the experiment (Run 1-6) in the same mesocosms after a priming period.

System Settings

All mesocosms received the same treatments and management practices within each iteration of the experiment except for changes in copper concentration, which we altered via aqueous cupric chloride (CuCl₂) addition. CuCl₂ was dissolved into 1L of reverse osmosis water and poured over each experimental container at the beginning of specific iterations at rates indicated in table S1. Control mesocosms received the same volume of water (1L) during the amendment period. Climate variables that affect N₂O fluxes were maintained as consistently as possible via the Argus Prime automation system and monitored continuously or weekly.

The experiment was conducted with large mesocosms (121 L containers, 55.88 cm diameter) to avoid pitfalls of small column experiments (e.g., edge effects, soil behavior, root impacts) that would influence N₂O fluxes. Each container received 8 maize seeds at close to standard agricultural densities (20 -30 cm apart) in Paxton agricultural soil. After 2 weeks, we thinned to 4 stalks, selecting the smaller stalk to be removed, when necessary.

In corn production, many farms apply high rates of nitrogen fertilizer (*I*), and N₂O fluxes are positively correlated with nitrogen application rates (2, 3). Because of this, we injected urea-ammonium-nitrate fertilizer (NPK 32-0-0; 7.75% ammonia-nitrogen, 7.75% nitrate-nitrogen, 16.5% urea-nitrogen) at a high rate of 242kg N/ha to ensure a visible N₂O flux peak and to represent industrial practices. To minimize risk of seed burning, the injection was 5 cm from the seeds.

The temperature was kept at 28°C in the day and 17°C in the night with a 15 hour photoperiod, representative of average July conditions in Spring Grove, Illinois, USA, as N₂O fluxes are sensitive to temperature (4, 5). The two experimental types of mesocosms were distributed throughout the greenhouse to avoid issues that could arise from spatial heterogeneity of these variables (fig. S1).

Because N₂O fluxes are sensitive to soil moisture (3–5), we aimed for an soil saturation of 60% (VWC of 25). We irrigated with 5oz of reverse osmosis water per day via automated irrigation lines and Netafim Netbow 10-in drip rings, and we allowed for drainage through eight 1.27cm holes covered by egg crate material and landscaping fabric.

This series of experiments can be considered a time series, as no new soil was added in between iterations of the experiment. Once the N₂O fluxes returned to baseline values, typically 24-29

days after fertilizer addition, we ended the experiment. At the end of each iteration of the experiment, the corn stalks were cut at ground level, to prepare for the next run. Each consecutive run received new corn seeds as described above. Each following run can be treated as a subsequent growing season on previously amended soil.

Measurements

Weekly measurements of soil moisture at 15 cm, 35 cm, and 50 cm; soil pH; and buffer pH were taken. Soil moisture (VWC) was taken with a Spectrum Technologies TDR 150 soil moisture meter with an accuracy of $\pm 3.0\%$ VWC. Soil pH (1:2.5 soil:water ratio) and Sikora buffer pH (6) were measured from surface soil samples, with a standard deviation of ± 0.024 pH units.

N₂O concentrations were continuously measured by a Picarro Cavity Ringdown Spectrometer model G2508. A 10-min measurement period was used with 10 Eosense automated soil flux chambers (eosAC-LT/LO) and an Eosense recirculating multiplexer (eosMX) (7–9). The Eosense software calculated the fluxes using a linear fit. A pre- and post-delay of 90 seconds was used to ensure high-resolution flux measurements and a maximum number of samples measured per day. A 5-min measurement period was used briefly after a crash in the system during Run 3 (07/13/2023–07/22/2023). The flux was still resolvable even with this temporary shorter measurement period. Continuous measurements of soil temperature were taken with the eosAC-LT/LO.

Copper concentrations (exchangeable fraction and solid phase) in the surface soil were determined at the beginning and end of each iteration of the experiment. The elemental concentrations were measured at the Yale Geochemistry Center (YMGC) on either an Agilent 8900 Triple Quadrupole inductively coupled plasma mass spectrometer (ICP-MS) or a Thermo Scientific Element XR ICP-MS, using a quartz spray chamber as the introduction system and indium solution as internal standard. To prepare the samples for analysis, approximately 100mg of soil was dried at 60–70 °C and then finely ground. The exchangeable fraction (EF) was extracted by adding 12mL of 1M ammonium acetate to the soil sample, centrifuging at 4500 rpm for 5 minutes and removing the supernatant. Aliquots of the EF samples were dried at 90 °C in pre-acid-cleaned Teflon beakers and subsequently digested with a mixture of 200 μ L of distilled nitric acid (HNO₃) and 600 μ L of distilled hydrochloric acid (HCl) to remove any organic matter, capped and left on a hotplate at 130 °C for 48 h, and evaporated to dryness. The samples were then reconstituted in either 1% or 5% v/v HNO₃ for concentration measurements on the Agilent or the Element, respectively. The remaining solid phase was then dried at 60–70 °C, ashed in a Thermo Scientific Linberg/Blue M box furnace at 600 °C for 24 h to remove organics and volatiles, digested in a total acid digest by adding 5 mL of distilled HNO₃, 5 mL of distilled HCl, and 1mL of trace-metal grade hydrofluoric acid (HF), capped, left on a hotplate at 100 °C for 24 h, and evaporated to dryness. The samples were then redissolved in 5 mL of 6N HCl. Aliquots of either 15 μ L or 10 μ L from these solutions were evaporated and reconstituted again in either 15 mL of 1% or in 4 mL of 5% v/v HNO₃ for concentration measurements on the Agilent or the Element, respectively. Each batch of samples was accompanied by a USGS certified reference material, SGR-1b shale, which received the same treatment as the samples and was routinely measured to evaluate the precision for the elemental analysis, which was within 7.6% (2.0% for Agilent) of the certified value. Based on routinely measured (every 10 samples) duplicates and replicates, the reproducibility for solid phase copper measurements was evaluated to be 3.9ppm (for priming and Runs 1–3 on the Element) and 2.0ppm (for Runs 4–6 on the Agilent) (average of 2.95ppm between priming, 1–3 and 4–6). For exchangeable fraction copper measurements, the reproducibility was evaluated to be 1.4ppm (for

priming and Runs 1-3 on the Element) and 0.14ppm (for Runs 4-6 on the Agilent) (average of 0.77ppm between priming, 1-3 and 4-6), respectively. Detection limits for solid phase and exchangeable fraction copper measurements were determined to be 0.031ppb (0.04ppb for Agilent) and 0.007ppb (0.01ppb for Agilent), respectively.

Soil multi-omics analyses

Soils for metagenomic and metatranscriptomic analyses were taken from the 0-10cm range in the mesocosms, for a range of timesteps (table S5). For the field trial analyses, soils were taken from 0-10cm depth in 2020 after 4 applications of ground basalt rock, at an annual rate of 50 t basalt ha⁻¹. Both DNA and RNA extractions and all sequencing were performed by the DNA Services Lab in the Roy J. Carver Biotechnology Center at University of Illinois at Urbana-Champaign. Total RNA was depleted with 80% prokaryotic rRNA probes and 20% yeast rRNA probes. Shotgun metagenomic and RNAseq sequencing were performed on two S4 NovaSeq X Plus lanes. Galaxy, an open source, web-based bioinformatics server was used to map the sequencing reads to protein-coding genes. First the fastq files were converted into fasta files. Then FragGeneScan (10) was used to find fragmented protein-coding genes in short reads with the Illumina model and an error rate of 0.01. Subsequently, the eggNOG v2 tool (11) was used for functional annotation of novel sequences using precomputed eggNOG-based orthology assignments. Next using the Diamond alignment tool (12) in amino acid blast alignment mode, we aligned the short amino acid sequences against the protein database RefSeq (2023-07-14) (13) with 1 as the maximum number of target sequences to report alignment for per query. Sequencing reads were normalized to genes per million (GPM) or transcripts per million (TPM) by (1) dividing the length of the query (in base pairs) by the total length (in base pairs) of the closest-matching subject in the RefSeq database followed by (2) a normalization to the total number of hits scaled up to 1 million.

To taxonomically annotate the *nosZ* and *moxA* gene reads in our metatranscriptomic samples, we developed a comprehensive database that contains all annotated *nosZ* and *moxA* genes from the total collection of 74,196 bacterial genomes available to date accessed through the AnnoTree web-server (<http://annotree.uwaterloo.ca/>) (14). Each of the compiled *nosZ* and *moxA* sequences contained detailed taxonomy tags following the GTDB taxonomic assignments (15), allowing for easy breakdown of taxa in our samples. Paralogous groups of sequentially similar protein-coding genes and the whole set of SwissProt genes were also included in the database to limit false positive hits. Finally, our metatranscriptomic sequencing reads were searched against the created *nosZ*-*moxA* database using Diamond in amino acid blast alignment mode. The taxonomy of each of the *nosZ*- and *moxA*-expressing communities per metatranscriptomic sample were compiled into an abundance table of genera. Finally, these genus-level count tables were clustered using non-metric multidimensional scaling (NMDS) operated with Jaccard dissimilarity index. The distance from each of the *moxA* communities to the average *nosZ* community was computed as Euclidean distance from the Jaccard-NMDS-generated x and y coordinates. The extent of coupling between the two communities was based on a negative conversion of Euclidean distances.

Experiment iterations and different settings

All iterations of the experiment were performed with the conditions described above, and exceptions are noted in table S6 and are described here. Some iterations of the experiment involved tilling practices as tilling increases soil oxygen availability and decreases soil organic carbon content, leading to decreased N₂O fluxes (16). At the beginning of iterations that involved a “tilling practice,” all containers received simulated disc tilling of the upper 10 cm of soil.

Data Analysis

Cumulative emissions were calculated by integrating the flux measurements for each mesocosm using the trapz function from scipy.integrate in Python. We used a two-tailed t-test function from scipy.stats in Python to test the statistical significance of the difference of means between the experimental and control mesocosms' cumulative emissions. In GraphPad, we performed one-way ANOVA tests with post-hoc multiple comparison corrections, with a two-stage linear step-up procedure based on (17), to determine the statistical significance of the regulation of each gene's correlation to copper concentration and soil pH.

A random forest machine learning framework (18, 19) was used to predict the instantaneous N₂O fluxes from the various levers in the experiment: copper concentration, soil pH, temperature, soil moisture measurements, and time since fertilization. For this analysis, all manual measurements (soil pH, buffer pH, VWC, copper concentrations) were linearly interpolated to line up with the continuous flux measurements. This machine learning technique allowed us to assess the relative importance of each parameter in driving variations in the N₂O flux, which may have high-dimensional nonlinear patterns that would be difficult to fully describe with traditional numerical methods.

The random forest model was constructed with the ranger package (20) in R and the entire dataset was split into training and testing data (75%, and 25% respectively). The training data is used to build a random forest model with an ensemble of 600 trees and a minimal node size of 3. This trained model's performance is then tested on the unseen test data. The relative importance of each variable in determining N₂O fluxes is determined by the permutation method within the model. We first calculate the prediction accuracy on the original data and again on perturbed data (in which a single predictor variable is perturbed at a time). We then determine the permutation importance for each predictor by calculating the difference between the two accuracy values and the random forest permutation importance for the predictor variable is given by the average difference among all trees.

To get an understanding of the scale of impact copper could have nationally, we calculated an estimate of the change in annual N₂O flux across the conterminous United States for a given change in bulk copper and/or pH. We first performed a multivariable nonlinear regression (degree = 2) using the sklearn package in Python on the cumulative N₂O emissions (integrated over the same number of days for each run), the average solid phase copper concentration for each mesocosm in the respective run, and the average soil pH for each mesocosm in the respective run. The regression had an R² of 0.304.

$$\text{Equation 1: } N_2O_{\text{emission}} = 41326.67 * [Cu] + 6775549.43 * pH + 52.01 * [Cu]^2 + -8851.71 * [Cu] * pH + -508189.17 * pH^2 + -21113184.93$$

We then compiled a conterminous United States dataset of water-based soil pH measurements from the National Cooperative Soil Survey (NCSS) (21), annual modeled N₂O flux data (22), and bulk copper concentrations in the top 5cm (23).

Point data for pH and Cu were interpolated in ArcGIS Pro using Ordinary Kriging with a spherical semivariogram model, with variable search neighborhoods. Copper was interpolated separately for all land covers and for cropland areas, with the cropland model using log-

transformed Cu values. All interpolated rasters were produced and snapped to the modeled N₂O grid for exact extent and alignment. The fit of each interpolation model was evaluated using leave-one-out cross validation. The RSME's were 0.73 for pH, 20.54ppm for copper across all land covers, and 11.99ppm for copper on croplands only. R-squared values for each were 0.96, 0.54, and 0.97, respectively.

We calculated an expected percent change in national N₂O flux for a series of given changes in pH and bulk copper by applying Equation 1 to the initial and changed values. To create our initial calculation, we brought all values in our national dataset that were below our lowest values of copper (21ppm) and pH (5.25) up to those values. We then simulated the change in N₂O fluxes that would occur due to increasing those values of copper and pH up to a series of values (30, 40, 50, 60, 80ppm; and initial pH, 6, 6.25, 6.5, 6.75, and 7, respectively; Fig. 1D, Fig. 1E, fig. S8). In the case that the predicted N₂O percent change would increase for a given site, we did not perform the simulated amendment at that site (i.e., no copper was added, and pH was not increased). The percent change was then applied to the modeled N₂O dataset from (22).

The effects, availability, and retention of Cu will vary widely based on soil type and climatic conditions (24–27). However, in some systems, Cu perturbations can last for decadal timescales, implying that any Cu-based intervention could have lasting impact (28–31). We calculated an estimate of \$/tonneCO_{2e} by dividing the cost spent on elevating the copper in each scenario (using a fertilizer cost of \$4.40/kg CuSO₄·5H₂O, an estimated depth of 2cm, and a soil density of 1.1 g/cm³), by the change in N₂O (in CO_{2e}, using a global warming potential of 273) assuming flux reduction remained consistent over a 10-year period (Fig. 1F, fig. S9). We provide a range of Cu amendment costs in table S4.

Supplementary Text

Priming

Prior to the experiments, the soil had been idle and dry. It experienced rapid stimulation upon receiving nutrients (CuCl₂ and UAN fertilizer) and water, with higher fluxes in the copper amended mesocosms before equilibrating (data S1, data S2). We attribute this to a priming of the system. During this period, the standard deviation of the N₂O emissions from the experimental groups was much larger than the control groups (fig. S2). The priming period also experienced larger average fluxes compared to those of subsequent runs (data S1, data S2).

Regression based on exchangeable copper

While the regression used for our national estimates was based on solid phase copper concentration, as shown in Equation 1, we also performed the regression on the exchangeable copper fraction using the same methods, reported below. This regression had an R² of 0.413.

$$\text{Equation 2: } N_2O_{\text{emission}} = 376609.36 * [\text{Cu}] + 8717878.92 * \text{pH} + 27520.75 * [\text{Cu}]^2 + -106343.58 * [\text{Cu}] * \text{pH} + -691788.16 * \text{pH}^2 + -26223513.57$$

Metagenomic and metatranscriptomic insights into the full nitrogen cycle

We performed global analyses of pH- and Cu-driven changes across the full N-cycle at our mesocosms, finding little evidence for additional auxiliary effects as the expression of other genes

were generally unaffected either by pH or Cu (Fig. 2K). A notable exception was the expression of the central dissimilatory nitrate reduction to ammonia (DNRA) genes, *nirBD* and *nrfAH* encoding enzymes converting nitrite (NO_2^-) to ammonia (NH_3), which exhibited increased expression under high pH (data S3). Stimulation of DNRA under high pH may be an auxiliary pH-driven mechanism regulating N_2O emissions since it reroutes nitrate (NO_3^-) towards ammonia rather than towards denitrification and N_2O generation. Our metagenomics and metatranscriptomics analyses highlight Cu-driven global changes in the number of copies and transcripts of central N cycling genes, but do not provide information on the possible post-transcriptional and post-translational modification that Cu and pH levels may mediate. Similar to pH modification of *nosZ* enzymatic activity, Cu amendment is likely to also augment the activity of *moxA* and *nosZ* as central Cu-containing enzymes (Fig. 2K).

Manganese and copper cycling

As copper transport is increased (under high [Cu]), Mn transport co-aligns with increases in expression of *moxA* (fig. S10, data S3). However, Mn-containing enzymes such as Fe-Mn containing superoxide dismutase *SOD2*, manganese catalase *ydbD*, *nrdA*, *E* are down-regulated (likely to avoid damaging the cell by mismetallation with Cu as opposed to Mn) (fig. S10, data S3). This creates a pool of unused Mn^{2+} that may be what *moxA* is using for the Mn^{2+} to Mn_2O_3 oxidation. At the same time the downregulation in catalases, which can also catalyse Mn^{2+} to Mn_2O_3 in addition to their function in detoxification of H_2O_2 , may also avoid the precipitation of Mn_2O_3 intracellularly and instead promote the extracellular Mn_2O_3 oxidation by *moxA*.

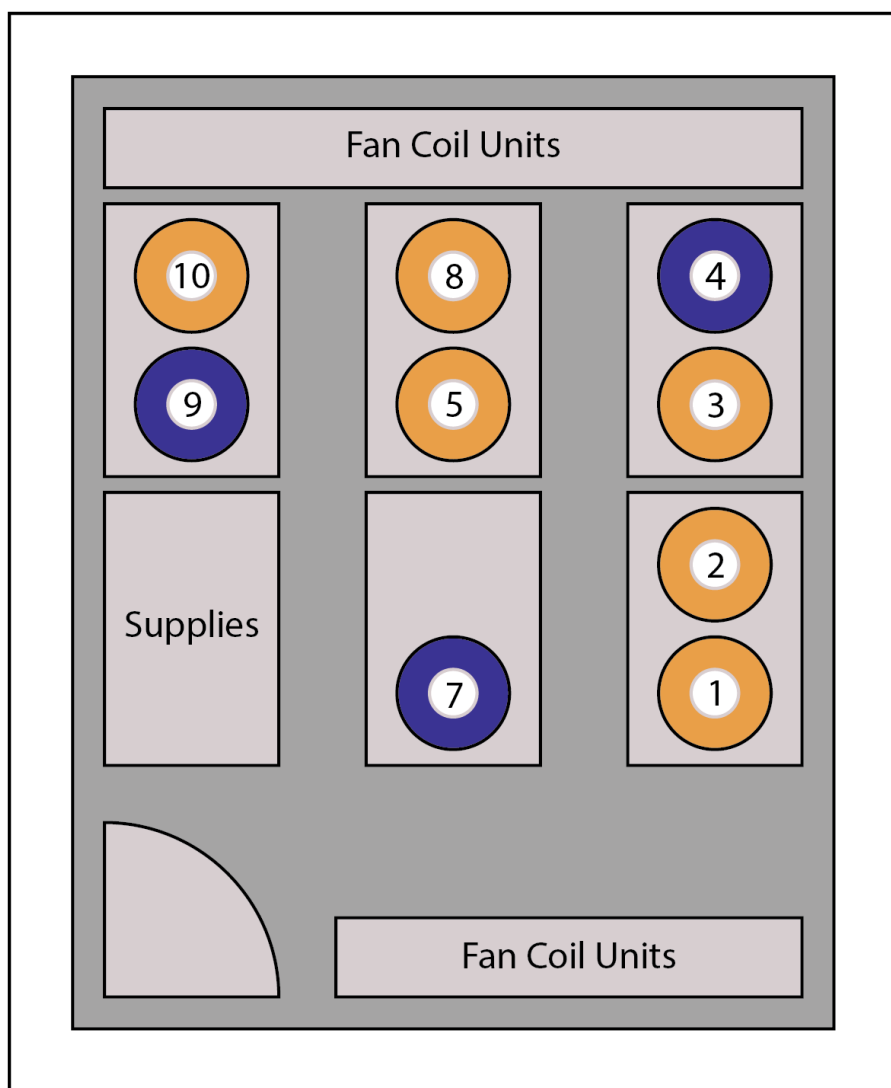


Fig. S1. Layout of research greenhouse. The layout with experimental mesocosms in orange and control mesocosms in dark blue. Mesocosms are labeled with numbers corresponding to their flux, manual, and omics measurements found in data S1, data S2, and data S3.

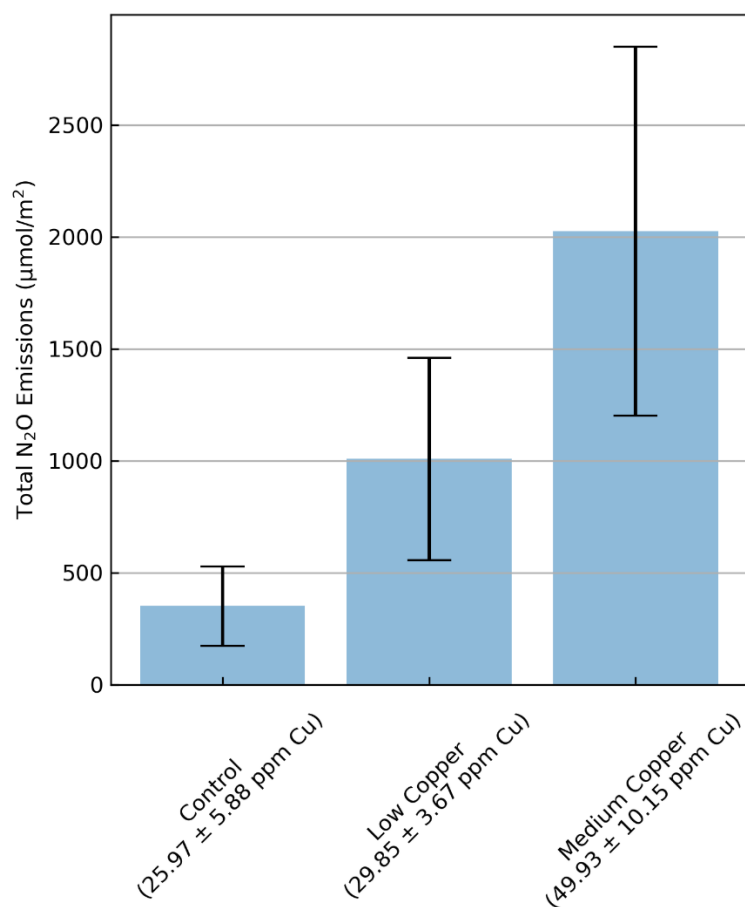


Fig. S2. Bar graph of cumulative N₂O emissions from Cu priming at beginning of experiment. Cumulative N₂O emissions (μmol/m²) calculated by integrating N₂O fluxes over time for the first 32 days after CuCl₂ addition and fertilization. Error bars represent 1σ standard deviation. Copper categories indicate the amount of CuCl₂ (in mg) that each experimental group received prior to measurements. Note that the soil had been idle and dry prior to the experiment, resulting in rapid nutrient cycling upon stimulation.

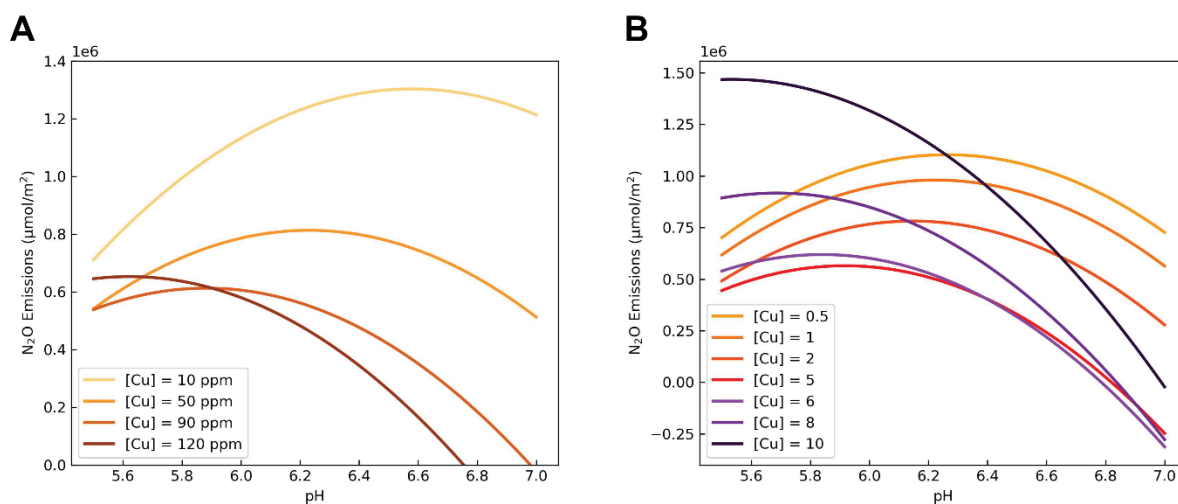


Fig. S3. Multivariable nonlinear regressions of Cu, pH, and N₂O. The regression, shown for (A) solid phase copper and (B) exchangeable fraction copper, is degree 2. It is color coded by copper concentration, with lighter colors representing less copper and darker colors representing a higher concentration of copper. Equations are shown in the methods and supplemental text.

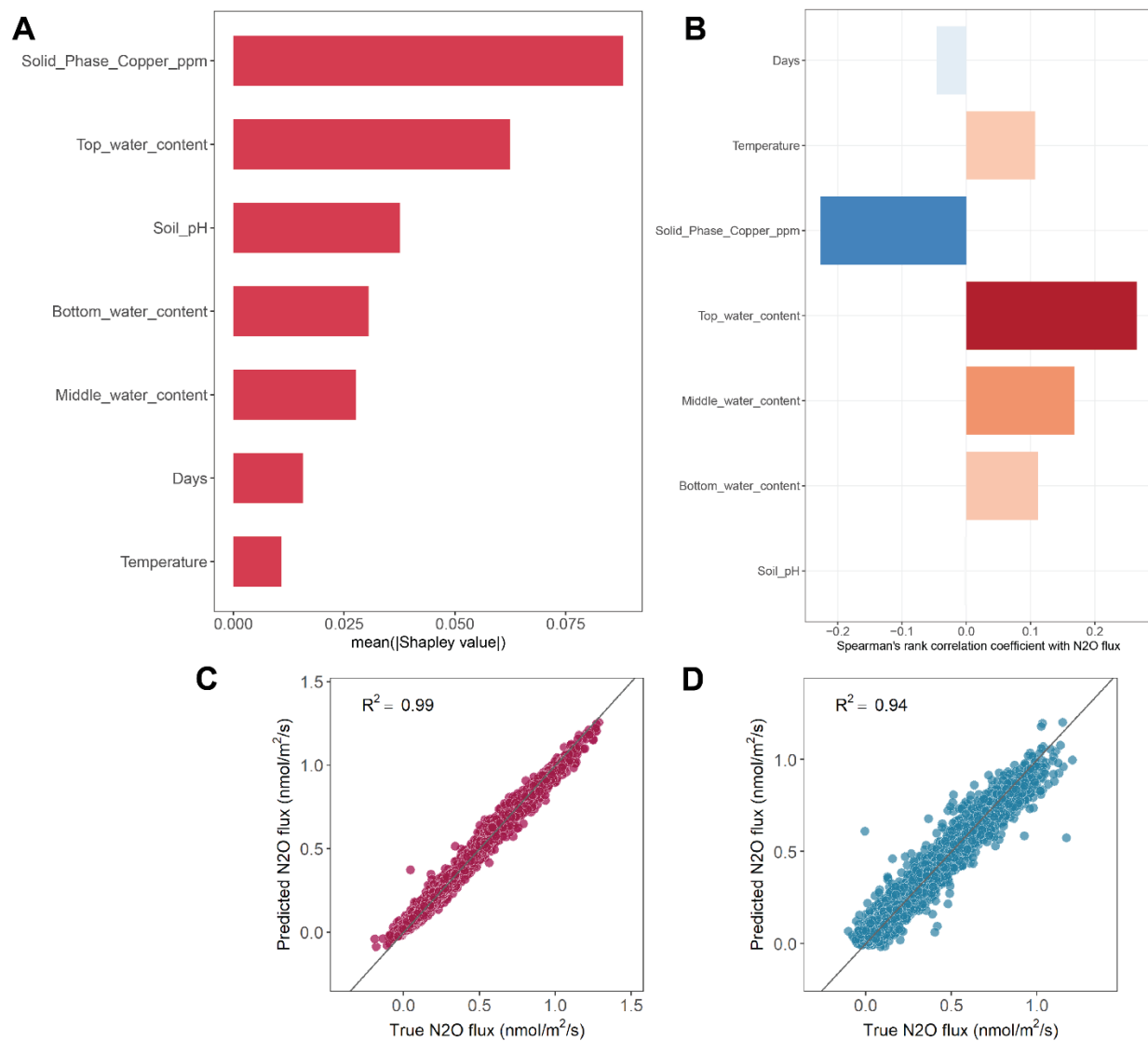


Fig. S4. Random forest machine learning results. (A) shows the SHAP-based importance of variables in determining N₂O fluxes with a larger Shapley value representing higher importance. (B) shows a spearman correlation to indicate whether variables are positively (red) or negatively (blue) correlated with N₂O fluxes. We show the R^2 for (C) training data and (D) test data.

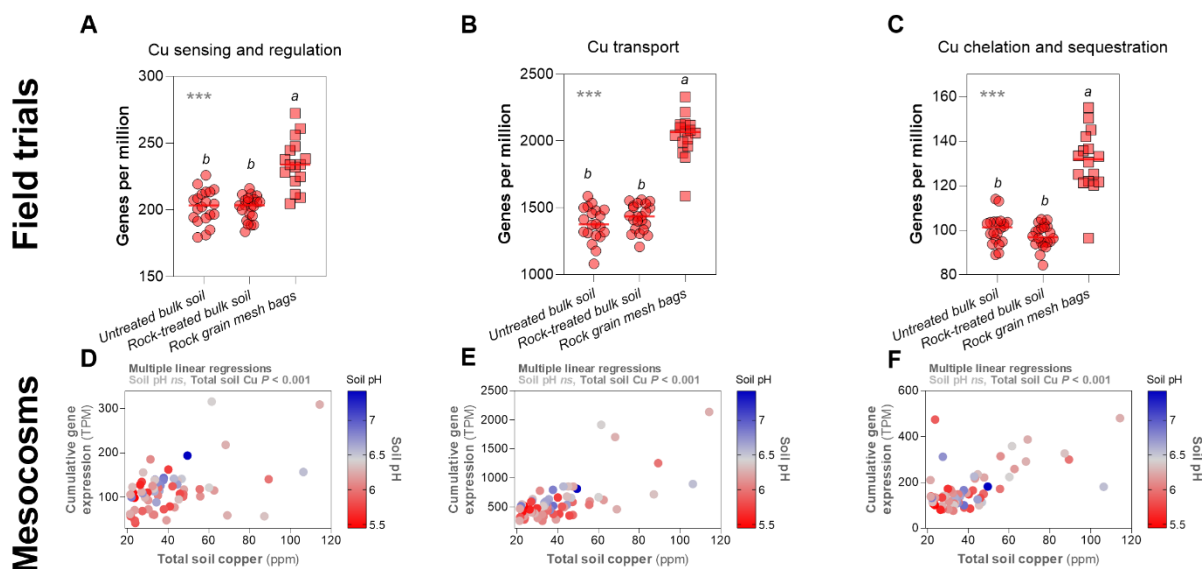


Fig. S5. Copper response in belowground microbial communities. Three major pathways of microbial response to copper are compared across enhanced rock weathering field trails (A-C) and mesocosms (D-F). Note that similar to copper amendment, rock powder mesh bags as microenvironments of high Cu availability, contained larger cumulative abundances of copper genes relative to soils. Statistical tests in A-C are ANOVA and these in D-F are multiple linear regressions. Asterisks of ***, **, and * represent p-values of < 0.001 , < 0.01 , and < 0.05 respectively.

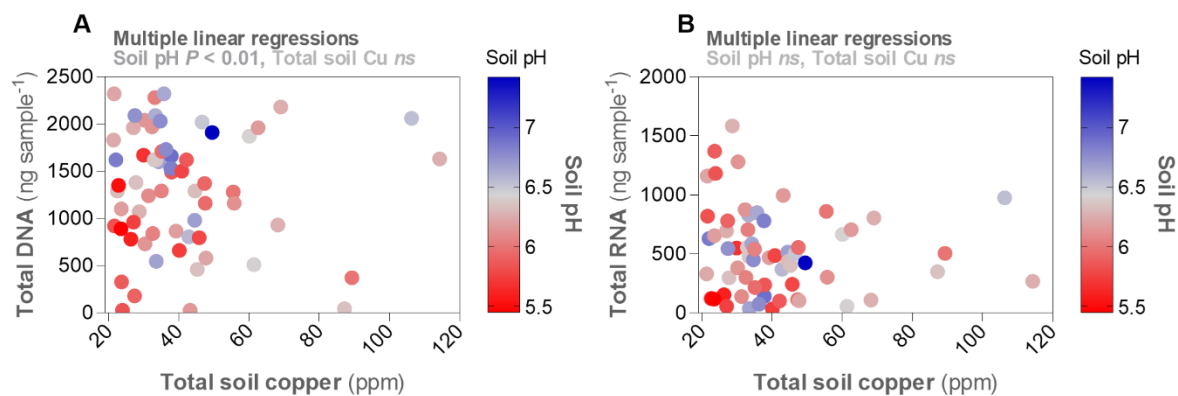


Figure S6. Total microbial DNA and RNA from mesocosm soil samples. Note that Cu did not impact the A. total DNA, nor B. the total RNA, indicating no antimicrobial effects on the bulk community.

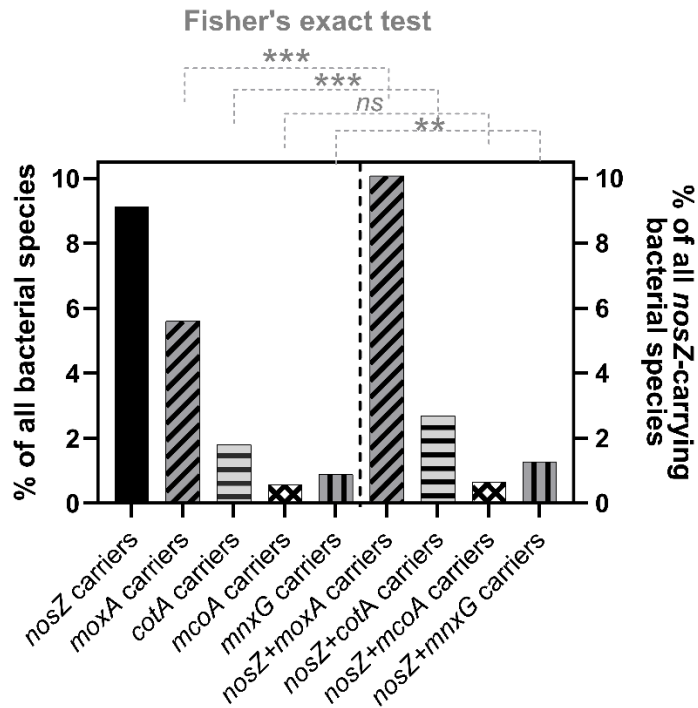


Fig. S7. Global multi-genome analyses of >74,000 bacterial genomes indicate that *moxA* is more frequently found in N₂O-reducing bacteria carrying *nosZ* than chance alone (calculated as the global average). Statistical tests are Fisher's exact tests. Asterisks of *, **, and * represent p-values of < 0.001, < 0.01, and < 0.05 respectively.**

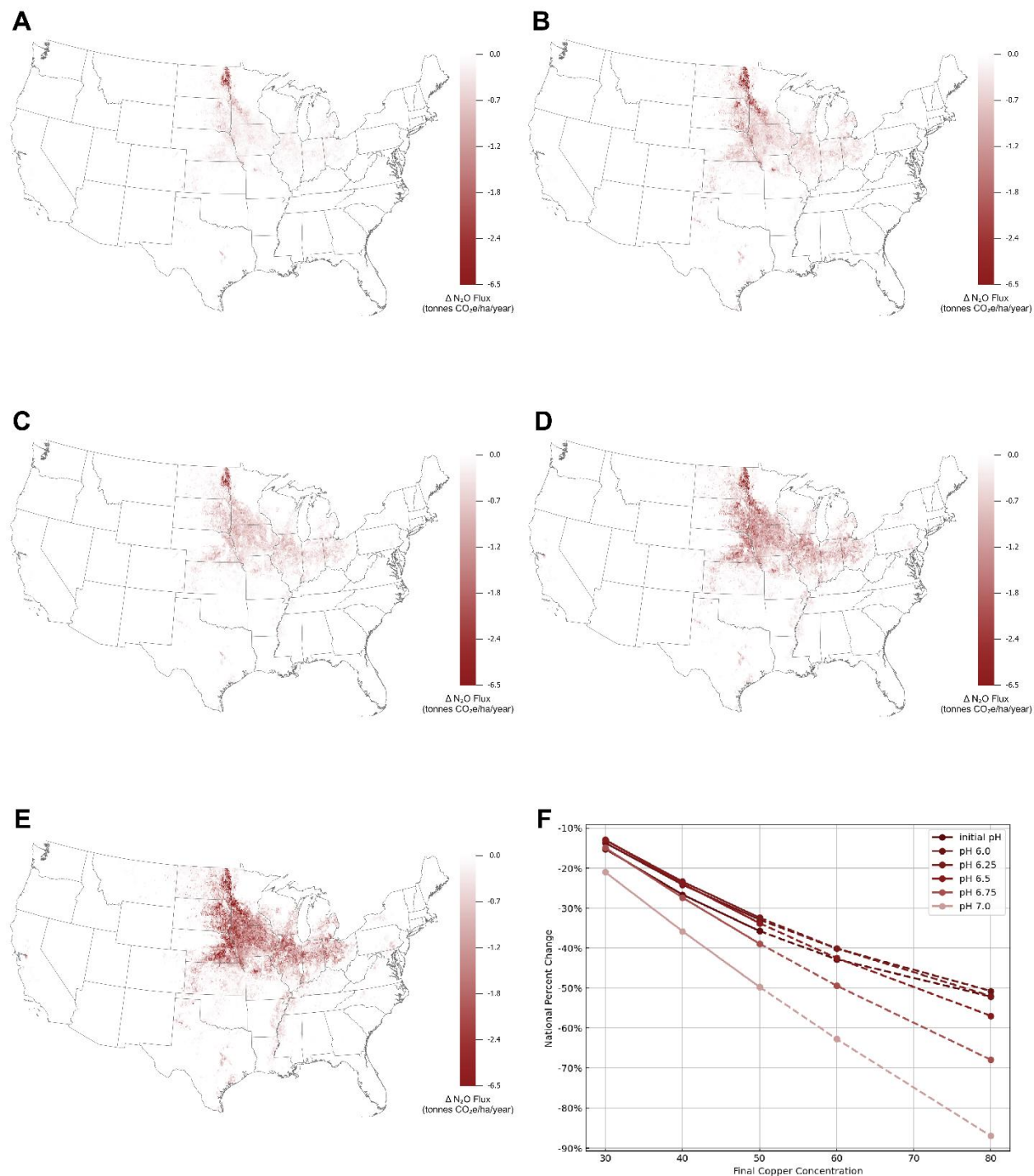


Fig. S8. Maps of Cu, pH, and N₂O fluxes, and changes in N₂O given Cu amendments in the United States. Tonnes of N₂O (in CO₂e) saved annually by Cu amendments on croplands to achieve a Cu concentration of (A) 30 ppm, (B) 40 ppm, and, by bringing the pH up to 7 with (C) 30 ppm (D) 40 ppm, and (E) 50 ppm. Shown in (F) is the national percent change in N₂O emissions annually given increases in soil Cu concentration on all land (not just cropland) to 30, 40, 50, 60, 80, and 100 ppm bulk Cu and increases in soil pH to none, 6.0, 6.25, 6.5, 6.75, and

7.0 when possible and determined beneficial based on the regression (Equation 1). Prediction lines are dashed past 50 ppm, at which point different nations have different Cu threshold recommendations.

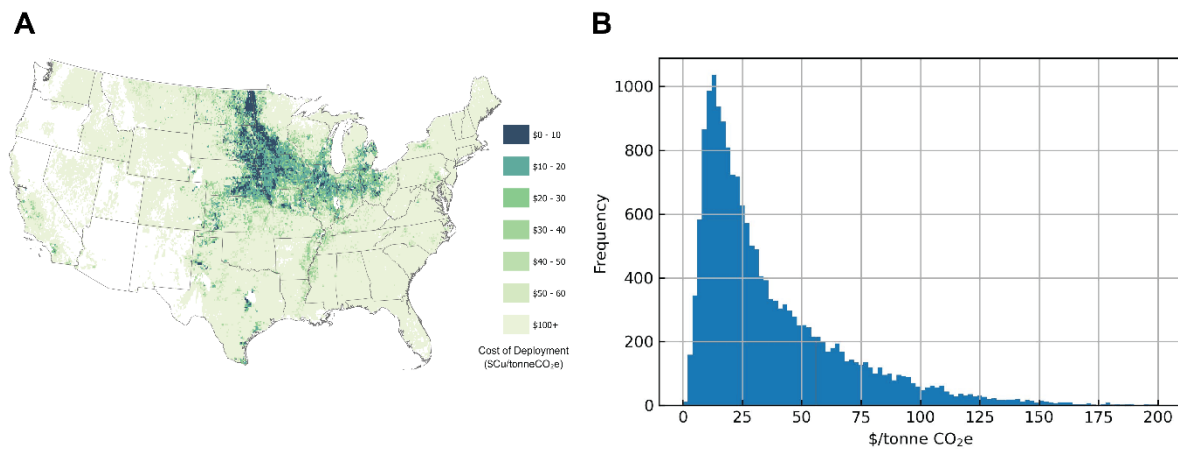


Fig. S9. Map and histogram of theoretical cost of deployment. The projected cost of Cu deployment on croplands per tonne of CO₂e mitigated at a rate of 30 ppm Cu, given no changes in pH in the form of (A) a map and (B) a histogram. The histogram is bounded by locations where the N₂O flux is greater than 0.1 gN/m²/yr and costs less than \$200/tonne CO₂e (i.e., where it would be a viable strategy). See methods for calculations.

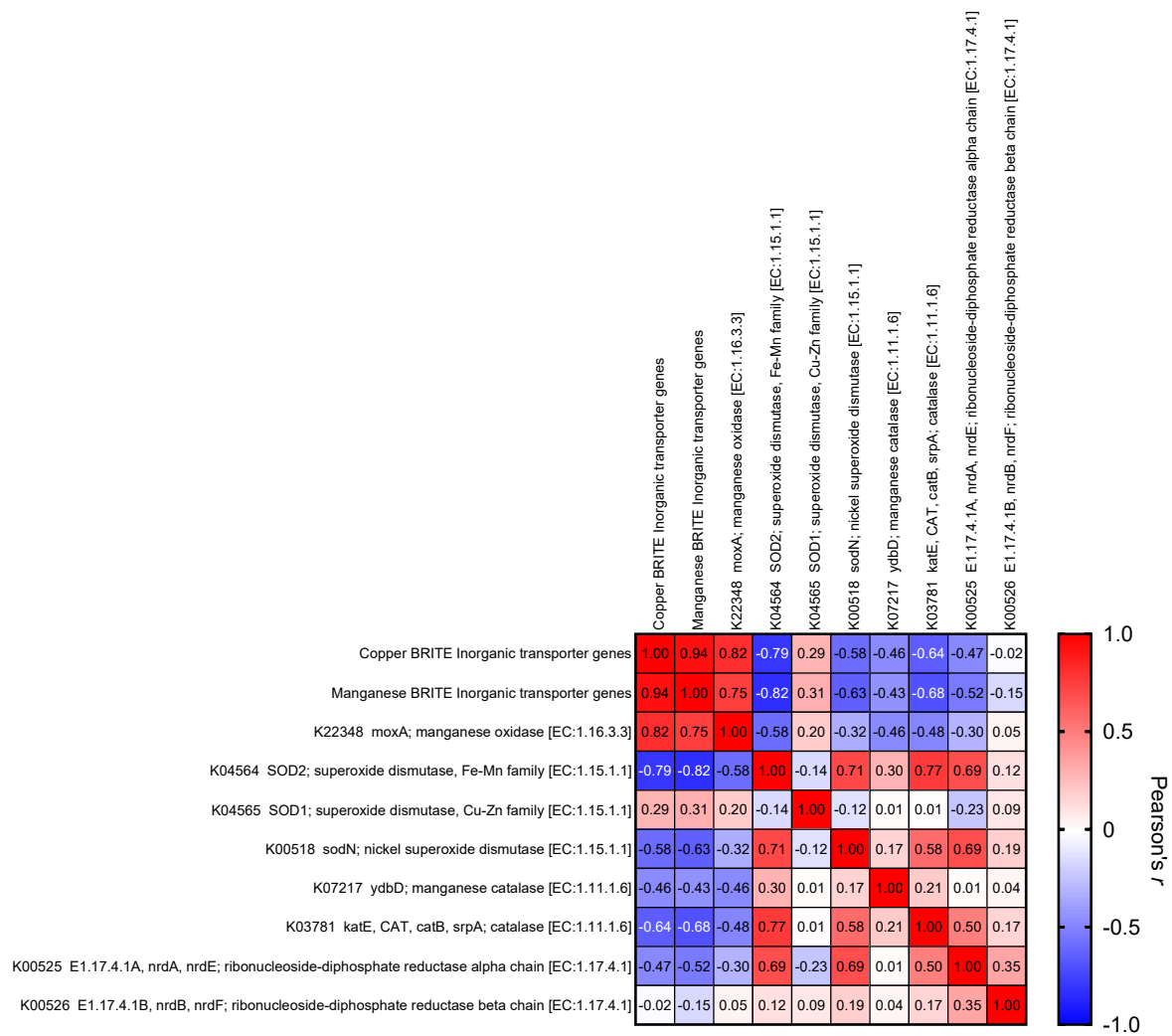


Fig. S10. Correlation heatmap of expression of genes within the mesocosm experiments. A heatmap of the Pearson's r associated with Cu- and Mn-cycling and transport genes in the mesocosm experiments.

		Pot 1	Pot 2	Pot 3	Pot 4	Pot 5	Pot 7	Pot 8	Pot 9	Pot 10
Priming	(mg Cu/pot)	123	35	123	0	35	0	123	0	35
	(kg Cu/ha)	5.9	1.7	5.9	0	1.7	0	5.9	0	1.7
Run 1	(mg Cu/pot)	0	205	123	0	83	0	0	0	204
	(kg Cu/ha)	0	9.7	5.9	0	4.0	0	0	0	9.7
Run 2	(mg Cu/pot)	0	0	0	0	0	0	0	0	0
	(kg Cu/ha)	0	0	0	0	0	0	0	0	0
Run 3	(mg Cu/pot)	0	0	0	0	0	0	0	0	0
	(kg Cu/ha)	0	0	0	0	0	0	0	0	0
Run 4	(mg Cu/pot)	314	198	194	0	317	0	317	0	201
	(kg Cu/ha)	14.9	9.4	9.2	0	15.1	0	15.1	0	9.6
Run 5	(mg Cu/pot)	0	0	0	0	0	0	0	0	0
	(kg Cu/ha)	0	0	0	0	0	0	0	0	0
Run 6	(mg Cu/pot)	0	0	0	0	0	0	0	0	0
	(kg Cu/ha)	0	0	0	0	0	0	0	0	0
Total	(mg Cu/pot)	437	438	440	0	435	0	440	0	441
Area integrated total	(kg Cu/ha)	20.8	20.8	20.9	0.0	20.7	0.0	20.9	0.0	21.0

Table S1. Cu (reported in both mg Cu/pot and area integrated as kg Cu/ha) added to each pot in the form of CuCl₂ during each iteration of the experiment. Note that each row represents the amount of Cu added during its respective Run and the same soil was used from the prior iteration, and therefore the total amount of Cu added is the cumulative amount per mesocosm.

Run	t-test statistic	p-value	Average pH of system
Priming	-1.952	0.0918	6.52
1	2.495	0.0413	6.49
2	1.213	0.2643	6.3
3	1.048	0.3294	5.90
4	1.378	0.2108	5.81
5	1.046	0.3302	5.41
6	2.603	0.0353	7.26

Table S2.

Statistics from a two-tailed t-test between the N₂O cumulative emissions from the experimental and control containers (note that the experimental containers had different levels of Cu). Bolded values indicate p-values of <0.05. A positive t-test statistic indicates that control containers had higher emissions than experimental containers, and a negative t-test statistic indicates that experimental containers had higher emissions than control containers.

Year	Control		Basalt	
	[Cu] (ppm)	Standard deviation 1σ	[Cu] (ppm)	Standard deviation 1σ
2016	17.6	4.4	18.6	3.8
2017	17.4	3.8	16.8	2.7
2018	16.5	5.2	17.8	2.9
2019	15.9	4.7	20.5	4.6
2020	18.0	4.0	21.6	3.5

Table S3. Solid phase Cu (ppm) in the Energy Farm field trials.

Region	Cost (USD/metric ton CuSO ₄)	Cost (USD/kg Cu)	Reference
United States	N/A	4.40	(33)
United States	2990	7.51	(34)
China*	2410	9.47	(34)
South America	2580	6.48	(34)
Southeast Asia	2500	6.28	(34)
Middle East	2730	6.86	(34)

Table S4. Cost of CuSO₄ amendments for bulk bags (November 2025; (33)) or industrial pricing (January 2025; (34)). *China is reported for CuSO₄-pentahydrate.

Run	Beginning/Fertilization Date	End Date	Omics sampling Date
Priming	12/16/2022	01/17/2023	01/17/2023
1	04/11/2023	05/08/2023	04/11/2023
1	04/11/2023	05/08/2023	05/08/2023
2	05/30/2023	06/27/2023	05/30/2023
2	05/30/2023	06/27/2023	06/13/2023
3	07/06/2023	08/01/2023	07/06/2023
3	07/06/2023	08/01/2023	07/20/2023

Table S5. Metagenomic and metatranscriptomic sampling dates for mesocosm experiments

	Priming	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6
Tillage performed at beginning of Run?	Yes	No	Yes	No	No	No*	Yes
Additional measurements?	Omics (end of run)	Omics	Omics	Omics	N/A	N/A	N/A
Copper added at beginning of Run?	Yes	Yes	No	No	Yes	No	No
Lime added at beginning of Run?	No	No	No	No	No	No	Yes (Rate: 6.7 tonnes/ha)

Table S6. Experimental system exceptions

*During Run 5, Pot 5 suffered an irrigation clog, and light-tillage was performed to resolve the issue and flatten surface of soil.

Data S1. (separate file)

Manual measurements for each Run for soil moisture (15, 35, 50 cm), electrical conductivity (15, 35, 50 cm), soil pH, Sikora buffer pH (Run 6), solid phase copper concentration, exchangeable fraction copper concentration, and biomass.

Data S2. (separate file)

Automated cavity ringdown spectrometer N₂O fluxes.

Data S3. (separate file)

Metagenomic and metatranscriptomic data, Pearson's r, and p-values.