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Article

Dynamics of Soil Microbial N-Cycling Strategies in Response to Cadmium Stress

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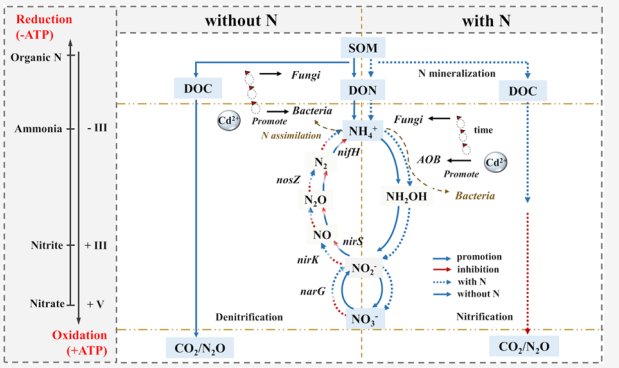
ABSTRACT: Globally increasing trace metal contamination of soils requires a better mechanistic understanding of metal-stress impacts on microbially mediated nutrient cycling. Herein, a 5-month laboratory experiment was employed to assess the effects of cadmium (Cd) on soil microbial N-cycling processes and associated functional gene abundance, with and without urea amendment. In non-N-amended soils, Cd progressively stimulated microbial populations for N acquisition from initial dissolved organic N (DON) to later recalcitrant organic N. The acceleration of N catabolism was synchronously coupled with C catabolism resulting in increased CO_2/N_2O fluxes and adenosine triphosphate (ATP) contents. The abundance of microbes deemed inefficient in N catabolism was gradually repressed after an initial stimulation period. We posit that enhanced exergonic N processes diminished the need for endergonic activities as a survival strategy for N communities experiencing metal stress. With urea amendment, Cd exhibited an initial stimulation effect on soil nitrification and a later a promotion effect on mineralization, along with an increase in the associated microbial populations. In N-amended soils, Cd accelerated N/C transformation processes, but decreased N_2O and CO_2 fluxes by 19 and 14%, respectively. This implies that under eutrophic conditions, Cd synchronously altered microbial C/N metabolism from a dominance of catabolic to anabolic processes. These results infer a nutrient-based adjustment of microbial N-cycling strategies to enhance their metal resistance.



KEYWORDS: *cadmium, nitrogen cycling, functional gene abundance, temporal effects, microbial resource allocation strategies*

1. INTRODUCTION

Nitrogen is an essential component of all living organisms. Microbially mediated nitrogen (N) cycling processes, such as N fixation, mineralization, nitrification, and denitrification, play critical roles in sustainable productivity, N_2O emissions, and eutrophication of aquatic ecosystems.[1,2] Anthropogenic disturbances, such as increased combustion of fossil fuels and growing input of nitrogen-based fertilizers and pervasive pollutants, continue to transform the global N cycle at a record pace.[3−5] In particular, the increasing accumulation of persistent pollutants such as heavy metals in marine and terrestrial ecosystems leads to a myriad of ecological and food safety impacts that have become urgent environmental issues.[6−9] These issues are especially relevant for agricultural soils as high inorganic N inputs may create positive feedback by increasing metal availability through nitrification-induced soil acidification.[10] Despite many efforts to understand the effect of metal toxicity on soil microbial N transformations, the ability to predict effects of metals is still limited. Investigations of metal-induced changes in microbial N-cycling processes under field conditions are limited by complex interactions among multiple soil physicochemical and biological factors.[11,12] Similarly, assessing the effects of heavy metals by modeling approaches fails to incorporate several real-world complexities.[13−15] Short-term laboratory experiments are conducted primarily for confirmation and comparison purposes, but these studies report that the response of microbial N-cycling processes was unpredictable, due to variability in the sensitivity of N-cycling microorganisms across different time-scales.[16−18] Thus, we still lack the necessary mechanistic understanding to develop a predictive framework of microbial response to persistent metal contamination and the subsequent consequences for ecosystem N-cycling.

Among persistent metal contaminants, cadmium (Cd) is one of the most toxic, ubiquitous nonphysiological metals, with an estimated biological half-life of 17−30 years.[19] Once internalized into biological systems, Cd could directly mediate DNA damage and lipid peroxidation,[20] or substitute essential cations (e.g., Fe, Ni, Co, Zn, and Cu) to perturb metal-

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loprotein function.[21,22] Biological strategies to mitigate metal toxicity remain widespread in unicellular organisms,[23] and are usually energetically expensive[24−26] and accompanied by cellular metabolic shifts.[25,27] Notably, some tolerant species have powerful resistance systems to survive under high Cd levels. For selected resistant species, the internal reallocation of energy necessary to sustain metal resistance mechanisms does not result in a shutdown of the nutrient processes they perform, especially for C and N metabolism.[27−30] Evidence suggests that successful microbial metabolic strategies for survival under extreme metal exposure progress toward the most efficient internal energy partitioning to mitigate metal toxicity.[29,30]

Usually metal-tolerant/resistant microbial taxa are selected within a few weeks or months in metal-contaminated soils, but a complete community adaptation to metal contaminants can take years or even decades.[31,32] As succession proceeds, adaptation processes including community reconstruction, proliferation of resistant populations, natural selection, and horizontal transfer of resistance genes usually impose a large energy cost[26] and lead to more pronounced alterations of nutrient cycles.[13,33,34] Thus, temporal dynamics must be considered in investigations of metal impacts on microbial communities. Given the strong control of a microorganisms' physiological costs over its internal nutrient (e.g., C and N) metabolism,[27−29] energy demands of the whole community are posited to determine the microbial composition and biogeochemical cycling of metal-contaminated ecosystems. However, disturbance-induced changes in microbial community activity are complex because they may occur via nonmutually exclusive mechanisms, including physiological stress responses, changes in the growth rate and turnover, and/or shifts in the composition of the microbial community.[35,36] Moreover, abiotic factors such as soil physicochemical properties and nutrient availability exert a strong influence.[37] This begs the question of whether Cd-contaminated soil ecosystems would naturally evolve toward a state that fosters maximization of energy harvest and nutrient exergonic bioenergetic reactions as succession proceeds.

Here, we posit that the response of biogeochemical N-cycling to Cd contamination is strongly determined by bioenergetic considerations. To assess this thesis, we conducted a 5-month greenhouse incubation (simulating a typical growing season timeframe) to investigate how Cd affects soil microbial communities and N-cycling processes. Soil samples were amended with or without urea to compare Cd effects under sufficient versus deficient N conditions (predominantly inorganic versus organic matter acquired N sources). As microbial N transformations are strongly influenced by microbial C:N stoichiometry,[38] we also examined Cd-induced changes in several C-cycling processes. Specifically, we focused on three main aspects, to (i) investigate the influence of Cd on soil mineralization and nitrification rates, N_2O and CO_2 emissions, adenosine triphosphate (ATP) contents, and relevant functional genes, (ii) compare Cd effects under predominantly organic versus inorganic sourced N, and (iii) elucidate the associated mechanisms mediating microbial N-cycling under elevated Cd levels. We hypothesized that (i) Cd would initially lead to an inhibition of soil N-cycling, due to immediate toxicity for a broad phylogenic range of microbes, but (ii) the inhibited N functions would recover over time due to the maintenance demands of the surviving species. We further hypothesized that (iii) the recovery of N-cycling processes will be faster with additional N inputs owing to increased nutrient and energy provisions. The results of this study provide information about soil nutrient management strategies to maintain soil health, food safety/security, and human/animal health from metal-contaminated soils.

2. METHODS AND MATERIALS

2.1. Experimental Soil. Soils were collected in December 2018 from a typical upland agricultural field, near the town of Wenling (28°21′ N, 121°21′ E), Zhejiang, China. Wenling has a subtropical climate with an average annual temperature of 17.3 °C and average annual precipitation of 1650 mm. Soils were sampled from the surface layer (0−15 cm) having a clay loam texture and USDA Soil Taxonomy classification of Alfisols. The fresh soil was mixed and sieved through a 2 mm screen with subsamples stored at −80 °C for DNA extraction or 4 °C for incubation experiments. Selected physicochemical properties of the experimental soil are listed in Table S1.

2.2. Experimental Design. To assess the impact of Cd on soil N-cycling, an aqueous solution of $CdCl_2{\cdot}2.5H_2O$ was applied to the soil to achieve added Cd concentrations of 4 and 8 mg Cd kg^{-1} dry soil. These Cd-contamination levels are equivalent to moderately and highly Cd-contaminated agricultural sites that need intervention according to the Risk Control Standards of China (GB15618-2018).[39] Soil without Cd addition (total Cd = 0.79 mg Cd kg^{-1} soil) served as an experimental control (reference state). Additionally, we examined Cd−N interactions by creating treatments with inorganic N amendment (with N) (soil amended with urea, an ammonia-based fertilizer, 150 mg N kg^{-1} soil) and without inorganic N amendment (without N) (inorganic N originating solely from soil organic N mineralization). Here, we choose urea as an inorganic N source owing to its common utilization as a cropland fertilizer in the world. Each treatment had three replicates, and each replicate had an associated subreplicate. One replicate sample contained 1000 g of soil for destructive sampling and the other contained 50 g of soil for the determination of CO_2 and N_2O fluxes throughout the experiment. Soil samples were placed in glass bottles and incubated at 25 °C in the dark utilizing a randomized incubation design.

To maintain constant water content at 60% of soil water holding capacity, moisture loss was compensated by the daily addition of MilliQ water. To assess changes in physicochemical and molecular properties, a subsample of soil was collected (50 g) at 0, 3, 7, 14, 28, 56, 91, and 154 days from the 1000 g of soil replicates. In sum, the experimental design followed 3 Cd levels × 2 N levels × 3 replicates × 8 sampling dates = 144 total samples.

2.3. Soil Mineral N Analysis and Quantitative Polymerase Chain Reaction (PCR). Soil-dissolved N was extracted with 1 M KCl (soil/KCl = 1:5, w/v). Total dissolved N (TDN) was analyzed with a Multi C/N TOC analyzer, NH_4^+-N and NO_3^--N concentrations were determined using a continuous colorimetric flow analyzer (San^{++}, Skalar, Netherlands). Dissolved organic N (DON) was calculated by the difference: DON = TDN − (NH_4^+ + NO_3^-).

Soil net mineralization and nitrification rates during a specific time period were calculated as: [(final dissolved inorganic N (DIN = NH_4^+-N + NO_3^--N)) − (initial dissolved inorganic N (DIN = NH_4^+-N + NO_3^--N) concentration)]/(incubation time)[40] and [(final NO_3^--N) − (initial NO_3^--N

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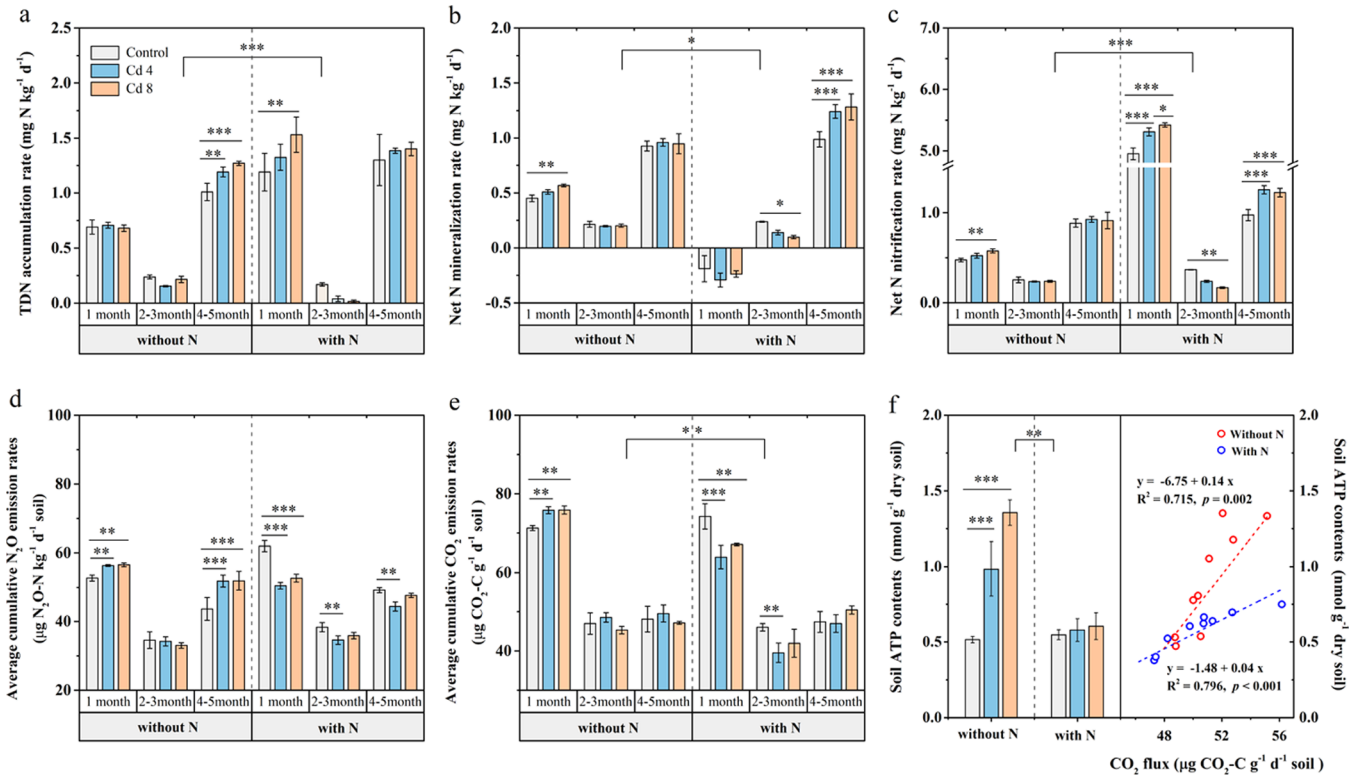

Figure 1. Effects of Cd contamination on nitrogen amended (with N) and nonamended (without N) soils for the (a) total dissolved nitrogen (TDN) accumulation rate, (b) net mineralization rate, (c) net nitrification rate, (d) cumulative CO_2 emission rate, (e) cumulative N_2O release rate, and (f) soil ATP content at the end of the incubation period along with its relationship with CO_2 flux. Mean ± standard deviation; $n = 3$; significant differences between individual Cd treatments and between N groups were performed by a one-way ANOVA and a pairwise comparison test, respectively, then complemented with a Turkey post-hoc test, asterisks indicate significance level: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

concentration)]/(incubation time),[41] respectively. The total dissolved N (TDN) fraction, determined as the sum of DON and DIN, originates from soil N depolymerization based on posited microbial N utilization pathways.[42−44] Hence, the TDN accumulation rate represents mineralization from recalcitrant N sources and serves as an important N-cycling metric, which was calculated as [net accumulation of total dissolved N (TDN = DON + NH_4^+-N + NO_3^--N)]/(incubation time).[45] Details of soil physicochemical analysis, measurement of soil N_2O, CO_2 fluxes, and ATP are described in the Supporting Information (SI). Specifically, we estimated the average cumulative CO_2 and N_2O release within a certain period via linear interpolation based on CO_2 and N_2O fluxes measured on each sampling dates and then divided by the entire incubation time to determine the average cumulative CO_2 and N_2O release rates.[46]

We performed quantitative PCR to quantify the abundance of phylogenetic and functional marker genes: bacterial *16S rRNA* and fungal *18S rRNA* (involved in N mineralization and assimilation), archaeal *amoA* and bacterial *amoA* (autotrophic ammonia oxidation), *nirK* (nitrite reduction), *nirS* (nitrite reduction), *nosZ* (nitrous oxide reduction), and *narG* (nitrate reduction) and *nifH* (N fixation) on soil samples taken after 3, 28, 91, and 154 days of incubation (see Text S1 for procedural details). These marker genes are generally classified as N transformation microbes: organic N-decomposers (nonspecific), nitrifiers, denitrifiers, and N-fixers.[1]

2.4. Data Analysis. Statistical analysis was performed using SPSS 18.0 (SPSS Inc.). Analysis of variance (ANOVA) was used to compare Cd treatment effects on soil response variables (e.g., net mineralization rate and net nitrification rate, TDN accumulation rate, cumulative CO_2 and N_2O release rates, and ATP content). A Tukey HSD pairwise comparison test was utilized for analyzing statistical significance among N treatments.

Response of microbial abundance to Cd treatment at each sampling date was reported as a ratio, which was calculated as the mean value of gene abundance in Cd treatments divided by the mean value in the Control. Log-transformation (Log 2 fold) was utilized for normalization of the microbial response to Cd. Simple correlation and multiple linear regression analyses were conducted using R software. Figures were prepared using Origin 9.1 (OriginLab) and R software.

3. RESULTS

3.1. Effects of Cd on Soil Net TDN Accumulation, Net Mineralization, and Net Nitrification. Compared with the control soil, microbial N turnover in Cd-contaminated soils was stimulated, with the magnitude and key processes differing with exposure time and Cd-contamination level. The TDN accumulation rate, which reflects potentially mineralizable N, did not respond to Cd addition until after 3 months and tended to increase with increasing Cd concentration (Figure 1a). Although net mineralization and nitrification rates were initially stimulated with the addition of Cd, the response persisted for only ∼1 month (increased by 13−26 and 10−21%, respectively) (Figure 1b,c). This indicates that Cd promoted N-cycling by utilization of DON to more recalcitrant N sources as the incubation period advanced.

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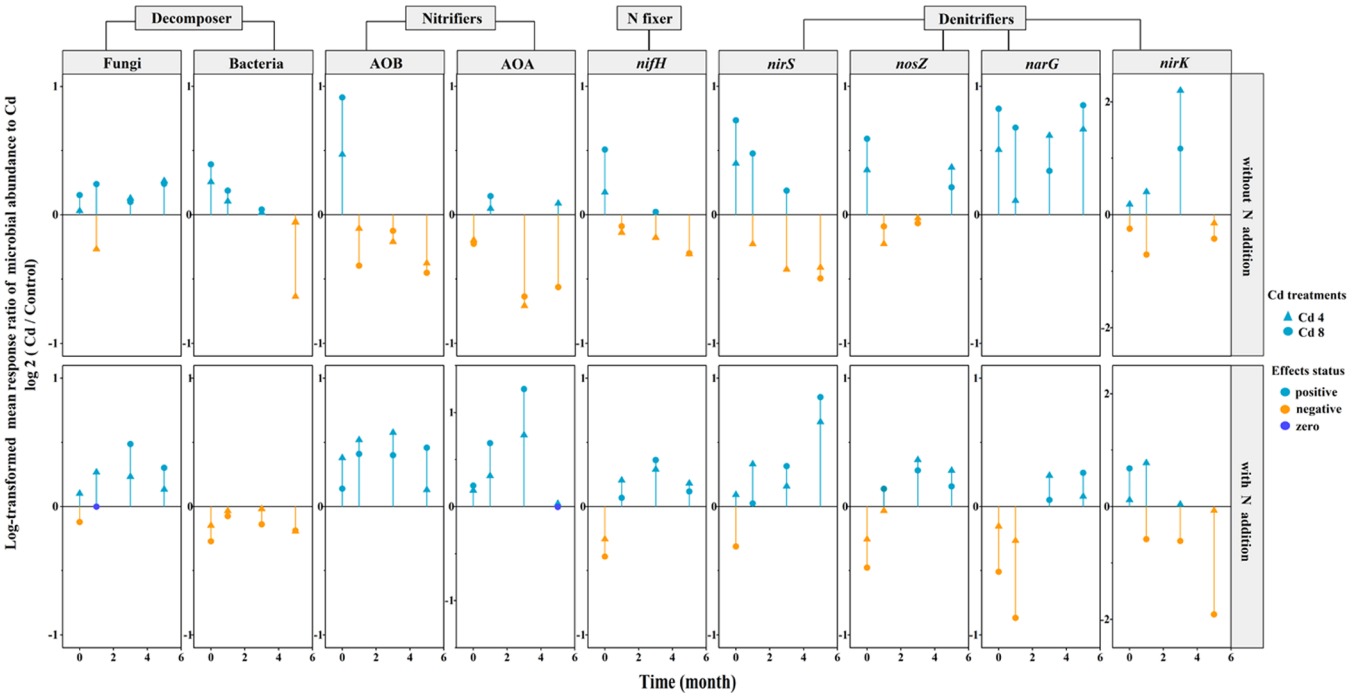

Figure 2. Log-transformed mean response ratios for the abundance of fungi, bacteria, and N-cycling microbes to Cd contamination in soils with or without N amendment. Values are for 3 days, 1, 3, and 5 months after Cd addition. Response ratios of microbial abundance to Cd treatment were calculated as the mean value in Cd treatments divided by the mean value in the Control. Log transformation (Log 2 fold) was utilized for normalization and standardization of microbial response to Cd.

Application of urea resulted in rapid soil nitrification and accumulation of TDN, but net mineralization was greatly inhibited. After the exhaustion of the initially large NH_4^+ pool, the inhibition of mineralization diminished (Figure 1a–c). Further, Cd addition initially (~1st month) stimulated net nitrification and TDN accumulation and later (4–5 months) promoted the net mineralization, inferring that Cd stimulated microbial N utilization from the dominance of inorganic to organic N sources over time. Notably, both soils with and without N amendment demonstrated stimulation of these N-cycling processes by Cd in the initial or later incubation stages that tended to increase with elevated Cd levels.

3.2. Effects of Cd on Soil N_2O and CO_2 Emissions and ATP Contents. Response of N_2O and CO_2 emissions to Cd addition was totally opposite in soils treated with and without N (Figure 1d,e). In non-N-amended soils with Cd addition, the average cumulative N_2O emission rates in the early (~1st month) and later (4–5 months) stages increased by 7 and 19%, respectively. In contrast, N-amended soils displayed a 15–19% decrease in cumulative N_2O emission rates following Cd addition (Figure 1d). This N_2O emission inhibition became moderated over time but was sustained throughout the entire incubation period. Notably, the promotion or inhibition of N_2O emission by Cd was not affected by the contamination level. The response of CO_2 emissions to Cd addition was highly consistent with that of N_2O emissions in soils with and without N amendment (Figures 1e and 3c,f).

We investigated the origin of CO_2 and N_2O emissions in Cd-spiked soil to elucidate the specific C/N-cycling steps that lead to their promotion or inhibition. First, we correlated CO_2 and N_2O fluxes with soil properties and found that the CO_2 release rate was significantly correlated with DOC (without N: $r = 0.50$, $p < 0.001$; with N: $r = 0.69$, $p < 0.001$), whereas N_2O release rate was correlated with mineralized N (DON + NH_4^+-N) (without N: $r = 0.66$, $p < 0.001$; with N: $r = 0.45$, $p < 0.01$) (Figure S3). Then, we correlated the response of C and N mineralization to Cd addition with the corresponding response of CO_2 and N_2O emissions (Figure S1). In non-N-amended soils, the Cd-induced increase of N mineralization and C consumption rates were nearly identical with that of the average cumulative N_2O and CO_2 emission rates (Figure S1a,b). In contrast, the N-amended soils displaying a large stimulation of mineralized N and C in Cd-spiked soils exhibited a sharp decline in the cumulative N_2O and CO_2 emission rates (Figure S1c,d).

Soil ATP contents significantly increased with Cd addition (Figure 1f). However, with N amendment, the promotion of ATP levels was sharply decreased and not evident. Linear regression analysis indicated a strong positive relationship between ATP contents and CO_2 release rates (Figure 1f).

3.3. Abundance of Bacteria, Fungi, and N-Cycling Functional Maker Genes. Quantitative PCR was used to estimate the number of bacterial *16S rRNA*, eukaryotic *18S rRNA*, and nitrogen-transforming genes in all treatments (Figure S2). We calculated the response ratios of their abundance to Cd and normalized (log 2 fold) these ratios for systematic evaluation (Figure 2). In non-N-amended soil (without N) with Cd addition, bacteria and almost all N-cycling microbes (except for ammonia-oxidizing archaea, AOA) were immediately stimulated. However, most of these stimulations gradually diminished and turned into inhibition during the later incubation stage. By contrast, fungi were not initially stimulated but became more prevalent with increasing incubation time (Figures S2 and 2).

Microbial response to Cd addition was also changed with urea application (Figures 2 and S2). Most microbes were significantly enriched by N input. However, under the inorganic-based N conditions (with N), the growth of bacteria,

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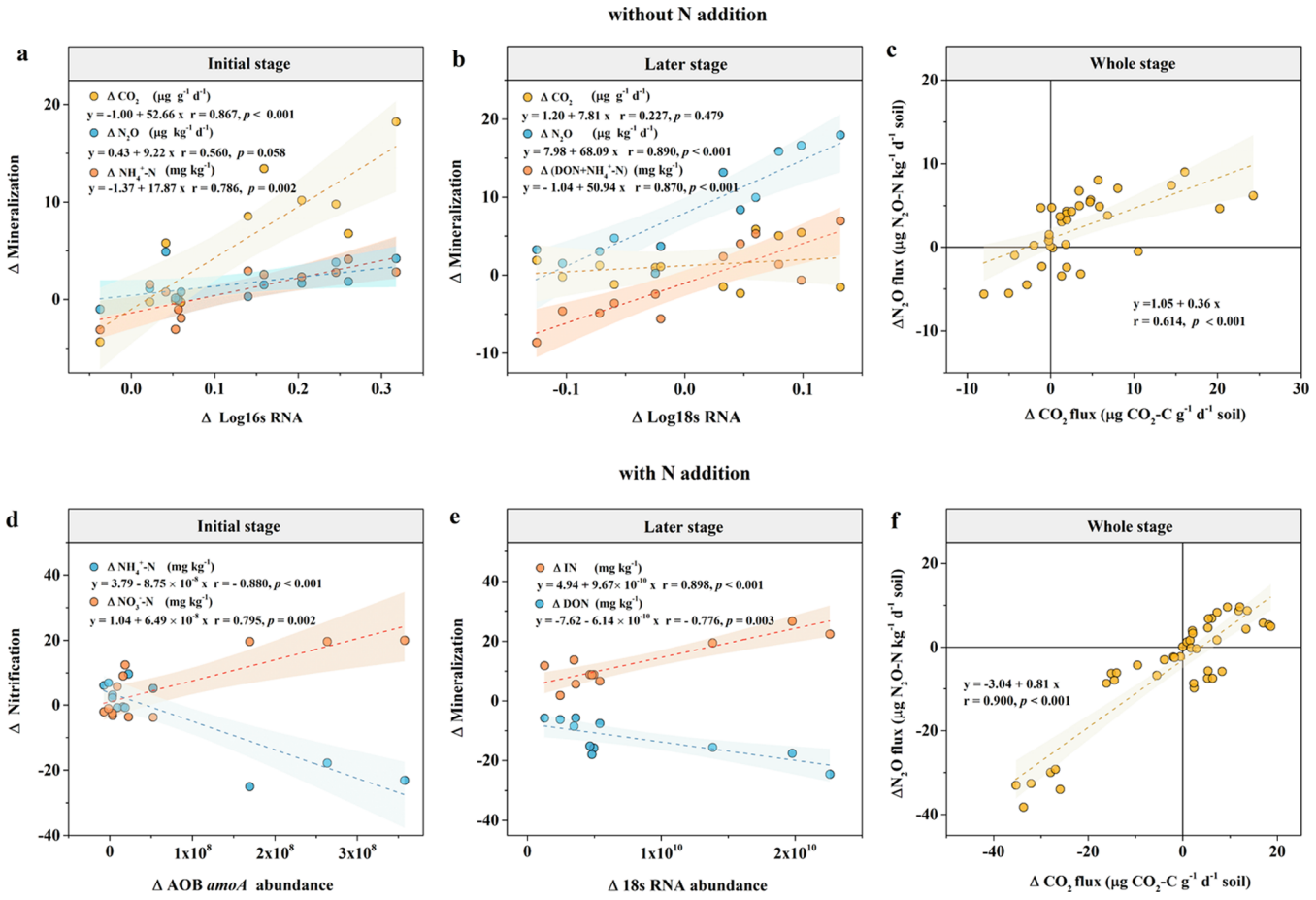

Figure 3. Relationship between the response of key soil N processes and associated functional gene abundance to Cd addition in soils (a, b) without and (d, e) with nitrogen (N) amendment at the initial stage (1st month) and later stage (4−5 months). The relationship between Cd effects on soil CO_2 and N_2O fluxes in soils (c) without N and (f) with N amendment during the whole incubation period. The responses of key N processes were assessed as the variations of corresponding N forms, while the microbial response was assessed as the mean absolute abundance difference or logarithmic difference between soils with and without Cd addition.

denitrifiers (*narG*, *nosZ*, *nirS*), and diazotrophs (*nifH*) was inhibited by Cd addition. Particularly, the inhibition of bacteria persisted throughout the whole incubation period, whereas the inhibition to other microbial components quickly disappeared and transitioned to a stimulation effect. In contrast, Cd addition consistently promoted nitrifiers (AOA and ammonia-oxidizing bacteria, AOB), while fungi were most strongly stimulated in the later stage.

3.4. Microbial Abundance versus the N Transformation Response to Cd Addition. We further sought to investigate the relationship between soil microbes and N transformations and to explore the specific microbial taxa regulating soil N transformations following Cd addition. Initially, we conducted a regression analysis of N-cycling processes as a function of microbial classes at different stages to serve as a primary sieve (Tables S3 and S4). Then we correlated the response ratios of the sieved microbial abundances with that of N processes to provide further confirmation (Figure 3). Carbon transformation (CO_2 emission) was also included in this investigation given the coupling between C and N-cycling processes.

For non-N-amended soils, the increase of bacterial and fungal abundance by Cd addition was strongly related to the promotion of N mineralization from DON (indicated by the increase of NH_4^+) in the initial stage (Figure 3a) and from recalcitrant N (indicated by the increase of NH_4^+ + DON) in the final stage (Figure 3b). Furthermore, these stimulations were accompanied by an increase in N_2O and CO_2 emissions reflecting greater overall microbial activities (Figure 3a,b). In contrast, Cd addition to N-amended soils increased the consumption of NH_4^+, and DON was strongly associated with the stimulation of AOB and fungi (Figure 3d,e).

4. DISCUSSION

4.1. Temporal Response of Soil N Transformation Processes to Cd Contamination. We posited that elevated Cd levels would induce a temporary inhibition of soil N-cycling due to immediate impairment (acute toxicity) of soil microorganisms,[12,47,48] followed by a gradual recovery of N-cycling functionality. Notably, we demonstrated that Cd additions led to more rapid N-cycling with the magnitude, key processes, and microbial mechanisms progressively shifting over time. In Cd-spiked soils, the net mineralization and nitrification rates were initially stimulated (~1st month), whereas the net TDN accumulation rate was stimulated at later time periods (4−5 months; Figure 1). This indicates that a five-month exposure period to elevated Cd induced an acceleration of soil N-cycling by driving microbial exploitation for N resources from DON to more recalcitrant N forms (Figure 1).

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Although several studies demonstrated negative effects of Cd on some soil microorganisms and their functions at lower metal loadings,[12,47,48] a toxicity threshold is expected to exist for individual microbial taxa and these toxicity levels may be site-specific (e.g., a function of soil physicochemical properties, such as pH and interactions with associated metals). Hence, the complex natural background of metals, soil biogeochemical properties, and initial microbial conditions interact to jointly determine whether the response to metal exposure will be net positive or negative.[16,49] Here, we found that Cd promoted N transformations through driving microbial exploitation for additional N resources from the soil. We attribute this finding to increased N demands for maintenance costs and production of relevant enzyme activities to cope with metal stress.[49]

Microorganisms under heavy metal stress utilize various resistance strategies to defend against toxicity, such as extracellular and intercellular sequestration, efflux pumps, enzymatic detoxification, etc.[26,50] The survival and growth of the selected species usually require more energy and are often accompanied by overall metabolic shifts.[26,28] An example is the transcription of genes related to amino acid biosynthesis and uptake pathways that were activated for potential protein detoxication.[27] Thus, to maintain these higher energy costs and synthesize enzymes, the exploitation and transformation of N might be expected to accelerate under Cd stress. As succession proceeds, microbial adaptation, either physiologically or genotypically[28,51,52] and either individually or communally,[31,32] inevitably imposes higher demands for energy and more irreversible metabolic transformations with time.[30,34] Therefore, our results infer that Cd-stimulated N-cycling did not diminish, but further increased over time by triggering N utilization from labile to more recalcitrant N forms as the incubation proceeded.

Exploitation of more N is generally coupled with the acceleration of C metabolic processes to obtain the energy to drive cycling processes.[53−55] Our study's enhanced microbial CO_2 emissions and ATP contents were consistent with higher N-cycling rates (Figure 1e,f). Homeostasis of microbial C:N stoichiometry supports this inference.[38] A higher microbial investment in C and N substrates is simultaneously required for the maintenance of energy and enzymes,[56] thereby causing a concomitant increase of N mineralization and C mineralization under Cd stress (Figures 1 and S1a,b). Other similar coupling of soil biogeochemical processes was also found in recent research.[57] As a consequence, N_2O and CO_2 emissions were synchronously stimulated (Figure 3c), implying linked production pathways between N_2O and CO_2. Furthermore, the concentration of solubilized C (DOC) regulated CO_2 emission, whereas mineralized N (DON + NH_4^+, rather than NO_3^-) levels were the most strongly related to N_2O emission throughout the entire incubation period (Figure S3a). This infers that N_2O originates from a broad range of processes (e.g., mineralization or heterotrophic nitrification) linked to CO_2 production, rather than the denitrification process. Autotrophic nitrification using mineralized NH_4^+ might also contribute to N_2O production. Similar coupled C/N pathways often occur in soils characterized by low inorganic N inputs and high organic matter content.[58] Thus, we posit that the Cd-induced higher exploitation of N is coupled with C resources (as an energy source) resulting in concomitant stimulation of N_2O and CO_2 emissions.

We attributed the progressive acquisition of N and C from labile to more recalcitrant sources following Cd addition to stimulated microbial populations. Our data suggest that N mineralization, as well as CO_2 and N_2O emissions, was more likely dominated by bacteria in the initial stage (1st month), whereas the later stage (4−5 months) was dominated by fungi (Table S3). Furthermore, a Cd-induced increase of bacteria and fungi abundance was accompanied by initial acquisition of N from DON and later attainment from recalcitrant N forms (Figure 3a,b). The microbial response to toxic metals is often characterized by a gradual population shift from bacteria to fungi.[59−61] Compared with bacteria, fungi are generally less susceptible to metal toxicity as exemplified by their slow positive increase of abundance in response to Cd addition in our study (Figures 2 and S2). Fungi are more resistant than other kingdoms to metal toxicity because they utilize diverse mechanisms of metal detoxification found in both prokaryotes (metal efflux pumps) and eukaryotes (intracellular sequestration).[62−64] In particular, intracellular sequestration is the most common tolerance mechanism employed by fungi and leads to chronic metal accumulation,[62,65] gradually affecting their growth and activity.[65,66] Bacteria are purported to utilize simpler nitrogen compounds, such as NH_4^+ and DON, whereas fungi are considered more effective at decomposing/utilizing more recalcitrant organic matter sources, such as chitin and proteins.[43] This likely contributed to the progressive boost of microbial N utilization over the course of incubation.

Concomitant with Cd selection for more efficient microbial strategies associated with C/N acquisition and energy harvest, the energy-limited soil ecosystem pursued strategies to minimize energy losses. For example, during the transition to the most efficient energy harvester groups dominated by fungi, the growth of less efficient bacteria [e.g., nitrifiers and denitrifiers and N fixers—energy consumers] were gradually repressed by Cd following an initial stimulation (Figures 2 and S2). Based on thermodynamic considerations, N mineralization is exergonic reactions, while denitrification is usually in need of exogenous energy provision or coupled to the oxidation of electron donors, N fixation is a thorough endergonic reaction (per molecule of nitrogen fixed, 16 molecules of ATP are consummed).[1] We attribute this inhibition to two primary mechanisms. First, following microbial acclimation to metal stress, higher maintenance demands imposed changes in resource allocation from growth to survival pathways.[30,36,67] Second, for inefficient C/N-acquisition and energy-consuming groups, the energy available was not sufficient to support their existing populations.[68] At the community level, these strategies theoretically support the hypothesis purporting the maximum energy harvest rate as the main selective pressure under extreme energy limitations.[68−70] Therefore, the response of soil microbial biochemical processes and functional community succession to metal stress might be predicable based on eco-energetic considerations.

In sum, the acceleration of N and C transformations by Cd addition was ascribed to a microbial resistance strategy in which specific nutrient cycling processes and key genes followed a time-dependent succession progression. The five-month exposure response to Cd-promoted soil N-cycling involved an initial stimulation of bacterial exploitation of DON followed by a later stage fungal decomposition of recalcitrant N forms. These N-cycling processes were coupled with enhanced C consumption as an energy source that led to synchronously accelerated N_2O and CO_2 emissions. Meanwhile, community energy-conserving strategies were also adopted to reduce some unnecessary costs, such as reductions in the population of

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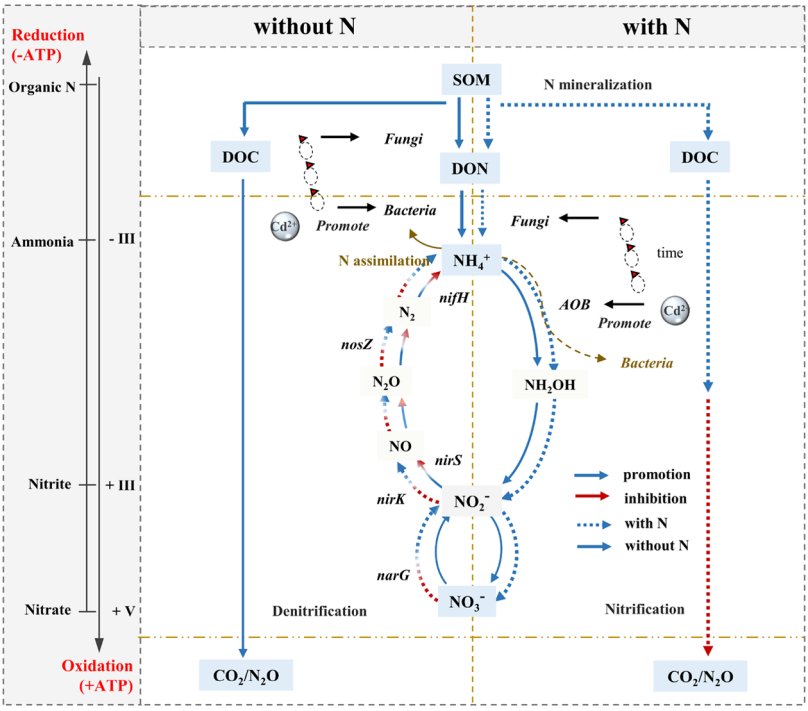

Figure 4. Schematic representation of microbial N strategies under Cd stress in soils with and without N amendment. In non-N-amended soils (without N addition), Cd-stimulated microbes, from bacteria to fungi, for N acquisition from the soluble to recalcitrant form with increasing incubation time. Exploitation of various N sources was coupled with higher C consumption causing an increase in CO_2/N_2O fluxes and ATP contents. Meanwhile, Cd gradually imposed an inhibition on the growth of microbes (bacteria and almost all N-cycling microbes) that were inefficient in N catabolism over time. Microbial survival strategies that enhanced exergonic activities and diminished the need for endergonic activities under Cd stress were magnified following urea addition (with N addition). Urea inputs supplied more accessible C/N resources either directly (fertilization) or indirectly (priming effect). To optimize community energy utilization, Cd-stimulated AOB-dominated nitrification at high NH_4^+ levels, which was subsequently supplanted by fungi mineralization as DON and recalcitrant N forms became the primary N source. Urea induced a shift of microbial C/N metabolism from the dominance of catabolic to anabolic processes in Cd-spiked soils, especially when NH_4^+ was at high levels for heterotrophic microbes. The energy-consumptive anabolic processes repressed other energy-expensive activities, such as bacterial reproduction (including denitrifiers and N fixers) and overall respiration, thus causing a decrease of CO_2/N_2O fluxes.

microbes that were inefficient with respect to N catabolic activities over time (Figure 4).

4.2. Response of N Transformation Processes to Cd Contamination Following Urea Amendment. The response of N transformations to Cd addition following urea amendment (with N) was sharply contrasting compared to the non-N-amended (without N) treatment. In N-amended soils, Cd-promoted soil microbial N utilization was gradually altered from the dominance of inorganic to organic N sources. However, enhanced N consumption and a corresponding increase in C consumption after urea addition did not induce an increase of N_2O and CO_2 emissions, but rather an inhibition.

Compared with the non-N-amended treatment (without N), the stimulation effects of Cd on soil N transformations were magnified by urea addition (Figure 1). We attribute this magnification to (i) an enlarged soil microbial biomass and (ii) increased accessibility of C/N resources after urea application. Almost all microbes (except for fungi) were significantly boosted by N treatment (with N) (Figure S2), which in turn imposed a higher demand for nutrient resources and energy by the increased microbial biomass. Furthermore, urea addition not only provided a large amount of NH_4^+, but also stimulated the decomposition of soil organic N (Figure 1a). This phenomenon, termed "priming effects",[71] indicates that inputs of N enhance C demands by microbes, thus accelerating soil organic matter (SOM) decomposition. These direct and indirect increases in C/N resources match the higher demands for C/N resources by microbes under Cd stress. Specifically, Cd exhibited an initial stimulation effect on the net nitrification and a later promotion effect of net mineralization (Figure 1), indicating an orderly alternation of microbial N utilization from the dominance of NH_4^+ to DON as the incubation period proceeded under eutrophic conditions (with N).

Other research has demonstrated that microbes from mining sites or heavy metal-contaminated habitats develop multiple N-source acquisition strategies to achieve full utilization of essential elements for support of metal detoxification mechanisms.[28,52,72] However, the genetic transformations necessary to develop multiple N source acquisition strategies usually take years to decades[31,32] and are difficult to achieve over the short timeframe of this study. Our results indicated a progression of N utilization from inorganic to organic dominance as microbial populations (from AOB to fungi) increased under Cd stress (Table S4 and Figure 3d,e). Thus, we posit that microbial N acquisition strategies observed in response to Cd stress were primarily C/N resource-driven.

Nitrogen inputs (with N) to non-Cd-contaminated soils simultaneously accelerated the consumption of DOC and the production of N_2O and CO_2. However, in Cd-contaminated soils, the enhanced N metabolism and C consumption was accompanied by reduced N_2O and CO_2 emissions (Figures 1 and S1c,d). Meanwhile, the stimulation of soil ATP contents was not obvious in Cd-spiked soils after N inputs (Figure 2f).

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These results infer that Cd drove microbial C/N metabolism from the dominance of catabolic to anabolic processes when accessible C/N sources were adequate. Diminishing respiration during growth arrest has been proposed as a microbial survival strategy.[73] From the transcriptomic level, microbes under Cd stress switched to an energy-conserving mode by inhibiting energy-consuming processes in favor of the production of stress-related proteins.[30] For example, *Enterobacter* was shown to lower its overall respiration under Cd stress to conserve energy for the synthesis of stress-related substrates, such as glutathione (GSH).[30] Moreover, heterotrophic bacteria, such as *Escherichia coli* and *Streptococcus*, switch from aerobic to anaerobic respiration for energy conservation[74] or intermediate generation[29] when exposed to Cd. These studies were mostly conducted in artificial media at nonlimiting nutrient levels. Here, under eutrophic conditions, it is possible for microbes to assimilate DOC with DON or NH_4^+ to synthesize additional stress-related substrates for metal detoxification purposes, rather than overall growth-related respiration expenditures.

Microbial energy-conserving strategies are not only confined to prohibiting overall respiration, but also involve repressing other metabolic processes that require high energy consumption, such as cell division.[30,74] This phenomenon is particularly apparent when accessible C and N resources are abundant. Our results indicated that with the exception of autotrophic nitrifiers, the abundance of bacteria and almost all N-cycling microbes was initially inhibited by Cd in the N-amended soils. By contrast, all of these microbes were immediately stimulated by Cd in the non-N-amended soils (Figures 2 and S2). We posit that this distinction is due to different microbial metabolism types under abundant NH_4^+ levels. The majority of denitrifiers, diazotrophs, and overall soil bacteria are heterotrophic and assimilate primarily ammonia for biosynthesis[1] of compounds essential for metal detoxification. This biosynthesis requires considerable energy consumption, thereby imposing a burden on more energy-expensive activities, such as cell division.[30,74] Therefore, their abundance was significantly inhibited by Cd when NH_4^+ was abundant. As the NH_4^+ source became depleted, organic N was preferentially utilized, this transition of microbial N turnover from anabolism to catabolism imposed an alternation of resource allocation from survival to growth pathways, thereby alleviating growth inhibition and leading to some stimulation (Figure 2).

In contrast, ammonia-oxidizing bacteria (AOB) and archaea (AOA) are obligate chemolithoautotrophs that oxidize ammonia to nitrite, producing a large amount of ATP[75,76] to fuel community survival. Thus, their growth was promoted by Cd stress when urea was co-amended. Specifically, AOB populations showed higher sensitivity to N amendment than AOA and were more likely to dominate nitrification processes, which is in accordance with previous studies.[48,77] Moreover, their internal resource allocation did not change with NH_4^+ depletion as abundant DON (legacy effect of urea priming) was mineralized to ammonia by soil enzymes before microbial uptake.[43] This allowed the Cd-induced stimulation of N-cycling effects to persist for longer time periods. Fungi, which are largely responsible for the decomposition of organic N, were more strongly stimulated by Cd in the later period due to their ability to more effectively utilize a wide range of organic N sources.

In sum, our results infer that the abundance of microbial groups with high efficiency for N catabolic processes was stimulated, whereas those groups involved primarily in anabolic processes were inhibited. This indicates that both energy-generating and energy-conserving strategies were adopted within the overall microbial community under conditions of Cd stress. To optimize community energy utilization through N catabolism, Cd-stimulated AOB-dominated nitrification at high NH_4^+ levels, which was subsequently dominated by fungi mineralization as DON became the primary N source. Under eutrophic conditions, Cd also shifted C/N metabolism from the dominance from catabolic to anabolic processes, the endergonic anabolic processes simultaneously repressed energy-expensive activities, such as overall respiration and heterotroph proliferation (Figure 4).

This study, for the first time, illustrated microbial N cycles from an eco-energetic perspective in a Cd-contaminated soil ecosystem. By characterizing the dynamics response of N-cycling processes and related genes to Cd contamination under both deficient and sufficient N source conditions, we demonstrated the temporal microbial mechanisms mediating soil N-cycling processes under Cd stress. Cd-induced progressive acceleration of microbial N catabolism and gradual attenuation of less energetically efficient microbial groups are postulated as efficient survival strategies for microbial communities experiencing metal stress under deficient N conditions. These survival strategies that enhance exergonic activities and diminish the need for endergonic activities were greatly magnified following urea addition. Enhanced C/N resource provisions improved community maintenance demands in Cd-contaminated soils, inducing an acceleration of N catabolic and anabolic processes simultaneously, thus determining the niche of the N-cycling microbial community under Cd stress. These results infer a nutrient-based adjustment of microbial N-cycling strategies to enhance their metal resistance. The results further suggest an evolutionary adaptation of soil nitrogen-transforming microorganisms toward the most efficient energy harvesting communities with selection determined by the integration of C/N-cycling processes providing the best energetic cost to metal toxicity benefit balance under the given environmental conditions. Under both deficient and sufficient N source conditions, the trajectory of N-cycling processes toward the most efficient energy harvest and minimizing populations of energetically inefficient or energetically consumptive microbial groups infers that soil biochemical cycles and functional community succession under persistent metal contamination may be predicable. Furthermore, the response trajectory also implies an intense competition for energy and resources among microorganisms, which may subsequently affect nutrient provisions for plants. Since our research is confined to phenomenological theory based on the description of research phenomenon and calculated relationships between microbial abundance and N processes, there is great potential for future studies to confirm metal-stress responses by examining molecular mechanisms. We also advocate for a further study incorporating ^{15}N isotope techniques to provide additional evidence to constrain quantitative estimates of individual process rates. Furthermore, microbial functional metabolisms in metal-contaminated ecosystems, especially regarding impacts on plant nutrients provisions, deserve more consideration.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c04409.

Details of soil physicochemical analyses, measurement of soil N_2O and CO_2 fluxes and ATP, methods of soil DNA extraction and real-time quantitative PCR; soil physicochemical parameters and qPCR primers with assay cycling conditions; multiple linear regression analysis of multiple N-cycling processes with microbial functional genes; dynamics of different forms of N, CO_2/N_2O flux, and functional gene abundance; relationship between the response of C/N mineralization and the response of N_2O/CO_2 to Cd; and relationship between microbial abundance N_2O/CO_2 and soil properties (PDF)

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Notes

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