Characterization of Natural and Affected Environments

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Organic carbon amendments affect the chemodiversity of soil dissolved organic matter and its associations with soil microbial communities

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Abstract
The “4 per mil” initiative recognizes the pivotal role of soil in carbon re-
sequestration. The need for evidence to substantiate the influence of agricultural
practices on chemical nature of soil carbon and microbial biodiversity has become a
priority. However, owing to the molecular complexity of soil dissolved organic matter
(DOM), specific linkages to microbial biodiversity have eluded researchers. Here, we
characterized the chemodiversity of soil DOM, assessed the variation of soil bacterial
community composition (BCC) and identified specific linkages between DOM traits
and BCC. Sustained organic carbon amendment significantly ($P < 0.05$) increased total
organic matter reservoirs, resulted in higher chemodiversity of DOM and emergence of
recalcitrant moieties (H/C < 1.5). In the meantime, sustained organic carbon
amendment shaped the BCC to a more eutrophic state while long-term chemical
fertilization directed the BCC towards an oligotrophic state. Meanwhile, higher
connectivity and complexity were observed in organic carbon amendment by DOM-
BCC network analysis, indicating that soil microbes tended to have more interaction
with DOM molecules after organic matter inputs. These results highlight the potential
for organic carbon amendments to not only build soil carbon stocks and increase their
resilience but also mediate the functional state of soil bacterial communities.
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Introduction

Soil organic matter (SOM) represents the largest pool (1500 ~ 2300 Pg C) of terrestrially organic carbon in the biosphere, this being more than two times the amount of carbon in the atmosphere. Hosting the largest diversity of organisms on land, soils play a pivotal role in regulating major global biogeochemical cycles (carbon, nutrients and water). As supporters of human food production systems, soils contribute to the economic status of nations. The role of SOM as an essential resource for heterotrophic life, its directing influence on soil food webs and the subsidiary effects it has on physical and chemical soil attributes (such as, structure, moisture content, nutrient availability, infiltration capacity) affirm SOM status as a key indicator of soil quality.

Despite the importance of the soil carbon reservoir, manifold pressures have resulted in substantial degradation of soil and SOM. A further antagonism to SOM depletion, of relevance to modern agriculture, is the use of inorganic fertilizers. With literature spanning almost a century, and field measurements, Mulvaney et al. evidenced chemical-N fertilizers ability to increased mineralization of native soil carbon. This enhancement in microbial utilization of SOC lead to demonstrable depletions of both soil carbon and soil nitrogen stocks. Depletion of SOC under continuous cultivation has led to the declines in crop yields in multiple cropping systems. The most obvious means to increase soil carbon is to augment new carbon, here numerous candidates (e.g. composts, animal sludges, sewage sludges, plant residues and biochars) have potential. Significantly, not only can these amendments assist in rebuilding soil carbon stocks, they also stimulate microbial populations, that in turn can influence the delivery of soil ecosystem services.
Dissolved organic matter (DOM) is an important constituent of SOM as it provides soluble organic substrates that support and sustain heterotrophic microbial communities. The variation of DOM composition regulates microbial growth and activity, and vice versa, therefore the association between individual DOM molecules and soil microbes lies at the heart of the DOM cycle. Our understanding of how soil microbes and DOM interact and the implications of these interactions for: soil microbial ecology, the carbon cycle, and the delivery of soil ecosystem services, are fundamental to our ability to use soils sustainably.

Only recently have tools emerged that can resolve the vast chemodiversity of DOM with analytical precision. Specifically, Fourier Transform Ion Cyclone Resonance Mass Spectrometry (FT-ICR MS) has paved the way for in-depth appreciation organic carbon profiles. Recently, this technique has successfully been used to characterize DOM chemodiversity in different environments, specifically, ocean water, lake water, soil pore water, sediment, and the atmospheric particulates. Several studies have characterized DOM chemodiversity in forest soils by FT-ICR MS, and they have shown that soil DOM chemodiversity changed with soil depth and significantly affected by soil pH and nitrate. However, few of these publications have provided any insight into the DOM chemodiversity and relationships between DOM and microbial communities in agricultural soils.

In this study we investigated the DOM molecular composition and bacterial community composition in an agricultural field with long-term fertilization. We characterized chemodiversity of soil DOM and analyzed its relationship with soil bacterial community. We hypothesized that the soil DOM chemodiversity significantly associated with soil bacterial community composition under long-term organic carbon amendments.
Materials and methods

Field experiment.

The soil samples were collected at experimental station (37°20′N, 116°38′E) of the Chinese Academy of Agricultural Sciences (CAAS), located in Dezhou, Shandong Province, China. The annual average temperature in the experiment site was 12.9°C, and the annual average rainfall was 522 mm. A long-term carbon-amendment/fertilizer experiment was conducted on an agricultural field with fluvo-aquic soil. The soil texture was clay loam, and the soil moisture capacity was 23%. The field experiment, from which samples were drawn, comprised randomized block of treatments (N = 3) maintained under a continuous rotation of winter-wheat/summer-maize for a decade.

Eight treatments were instated (Figure S1). Detailed information of the application rates of fertilizers was shown in Table S2. The fertilizers of phosphorus and potassium were applied as basal fertilizers. Annually (each June), all plots received the same application of basal fertilizer (superphosphate (600 kg hm$^{-2}$) and potassium sulphate (240 kg hm$^{-2}$)). Control treatments (CK) received basal fertilizer but no further carbon and nitrogen amendment. While additional inorganic fertilizer (0.5N and 1N), in the form of urea, was applied at 65 kg hm$^{-2}$ (0.5N) and 130 kg hm$^{-2}$ (1N) to provide low and high inorganic fertilizer regimes, respectively. To establish sewage sludge augmented plots, urea was again applied at the lower application rate (65 kg hm$^{-2}$) and sewage sludge applied (dry weight equivalent) at 4.5 t hm$^{-2}$ (0.5SS), 9 t hm$^{-2}$ (1SS), 18 t hm$^{-2}$ (2SS) and 36 t hm$^{-2}$ (4SS). Finally, plots containing chicken manure were established with urea applied at the lower application rate (65 kg hm$^{-2}$) and chicken manure applied (dry weight equivalent) at 10 t hm$^{-2}$ (CM). Thus, the CK and 0.5N treatments provided points of reference to discern SOC and BCC shifts directed by, i) urea fertilizer and ii) carbon amendments. In each plot, the fertilizers were spread over
the fields and mixed well with the soil (0 - 15 cm) immediately following the application. Sewage sludge (SS) was collected from Beijing sludge disposal plants, and then underwent composting. The chicken manure (CM) was purchased from a fertilizer company (Hebei Beautiful Day Fertilizer Technology Co., LTD.) in Hebei Province. Soil samples were collected in 2015 (ten months after their annual fertilizer and/or organic carbon amendments were added). Physical and chemical properties of soil, sewage sludge and chicken manure are provided in Table S1. Surface soil (0-15 cm) samples were collected from plots (~ 2 kg per plot). Each soil sample was a mix of ten soil cores per plot. After sampling, soil samples were immediately transported to laboratory on dry ice and stored at -80 °C.

Chemical characterization.

The pH of soil was measured in a solid-to-deionized water ratio of 1:2.5 using a digital pH meter (PHS-3C, Shanghai Lida Instrument Company, China). Total carbon (TC) and total nitrogen (TN) contents were determined by dry combustion in an element analyzer (Vario EL III - Elementar, Germany). Briefly, 5 g air dried soil was placed in a centrifuge tube (50 mL), and 25 mL Milli-Q water (18 MΩ) was added (1:5 solid:liquid ratio). The tubes were shaken in the shaker (170 rpm) and were centrifuged at 2,800 × g for 10 min. The supernatant was filtered (0.45 µm) and kept in the 4 °C until the determination of DOC and DTN concentrations by a TOC analyzer (Liquic TOC - Elementar, Germany). Subsamples were extracted with 2 M KCl solution, and the concentration of nitrate and ammonium were determined using a continuous flow analyzer (SAN++, Skalar, Holand).

Dissolved organic matter (DOM) analysis.

The solid-phase extraction of dissolved organic matter (SPE-DOM) from soil was performed as described in our previous work. More specifically, soil DOM was first
extracted with Milli-Q water (1:5 w/v) on a reciprocal shaker (170 rpm) for 8 h. Samples were then centrifuged at 2,800 × g for 10 min, and the supernatant were filtered through a 0.45 µm mixed cellulose ester membrane. SPE cartridges (Bond Elut PPL, 500 mg, 6 mL, Agilent Technologies) were activated by sequentially rinsing with pure methanol (mass spectrometry grade) and 0.01 M HCl. Acidified DOM samples (pH = 2) were passed over the activated cartridges and then the cartridges were rinsed with acidified Milli-Q water (pH = 2). After the cartridges were completely dried with ultrapure N₂ gas, DOM was eluted from the cartridges with methanol (5 mL) and stored at -20 ºC. Extraction efficiencies were calculated by drying methanol eluate and re-dissolving with ultrapure water. The extraction efficiencies were different among treatments (48 ± 11.2%, 65 ± 20%, 49 ± 18% and 50 ± 17.6% on average for CK, N, SS and CM). The final concentration of DOM was about 20 mg/L by methanol dilution. The molecular composition of solid-phase extractable DOM (SPE-DOM) was analyzed using a 9.4 T Bruker apex-ultra FT-ICR MS equipped with an electrospray ionization source (Bruker Apollo II) applied in negative mode. SPE-DOM was dissolved in methanol and injected into the electrospray source at 3 μL min⁻¹ by a syringe pump. PPL extraction blanks and solvent blanks were prepared and analyzed to check for possible contamination. Contaminated peaks in these blanks were removed from obtained DOM profiles. Detected mass peaks with S/N less than 6 were not considered in the following data processing.

Bacterial community composition (BCC) analysis.

DNA was extracted from soil (0.50 g) using the FastDNA Spin Kit for soil (MP Biomedical, Santa Ana, California, USA). 16S rRNA gene Illumina sequencing was performed on an Illumina Hiseq2000 platform at Novogene (Beijing, China). Community DNA was amplified utilizing amplification primers F515 (5'...
GTGCCAGCMGCGGGG-3’) and R907 (5’-CCGTCAGTTTGCAGTTT-3’) targeting the V4-V5 region of the 16S rRNA. The protocol described in our previous work, was adopted to process and analyze the obtained sequenced data. In summary, raw reads were filtered, quantified, and subsequently analyzed using QIIME. Operational taxonomic units (OTUs) were defined at the level of 97% similarity. The alpha diversity index and β-diversity were calculated based on operational taxonomic units (OTUs) table as described in our previous work.

**Statistical analysis.**

Absolute peak intensities of the FT-ICR MS spectra were normalized to the sum of peak intensities of a given spectrum, and thereafter referred to as *relative* peak intensities in the following statistical analyses. Compound groups were delineated by the following parameters according to previous studies: elemental ratios, aromaticity index (AI), double bond equivalence (DBE), and H/C cutoffs. A one-way analysis of similarities (ANOSIM) was performed (using R version 3.3.3) to determine if different treatments resulted in significantly different DOM molecular composition. The diversity index was calculated with R version 3.3.3 package ‘vegan’. Non-metric Multidimensional scaling (NMDS), based on the Bray-Curtis distance, was performed to evaluate the overall pattern of DOM molecules among the treatments. Correlations among DOM community, BCC and environmental variables were established using a Mantel test and redundancy analysis (RDA). NMDS and RDA were performed using R version 3.3.3 in the ‘vegan’ package. Linear discriminant analysis (LDA) effect size (LEfSe) was performed (using software sourced at: http://huttenhower.sph.harvard.edu/lefse/) with LDA set at > 2.0, to indicate significant difference in DOM chemo-markers among treatments.

The BCC was expressed in terms of relative abundances of OTUs for each of the
phylogenetic resolutions from phylum to species. Non-metric multidimensional scaling (NMDS), based on Bray-Curtis distance, was used to compare BCC profiles among treatments. For alpha diversity, the metrics of observed species (i.e. OTUs), Chao 1, and Shannon index were calculated. RDA analysis was conducted to determine the significant environmental parameters that shaped soil BCC.

The specific links between DOM chemodiversity and BCC were revealed by network analysis. Pairwise correlations were calculated using the ‘psych’ package in R version 3.3.3 to determine the relationships between individual DOM molecule and bacterial OTUs using Pearson product-moment correlation ($p < 0.05$). $P$-values were adjusted according to the false discovery rate to correct for multiple correlations. Where correlations revealed a pairwise Pearson’s correlation coefficients $R > 0.6$ they were considered statistically robust. These values were then taken forward and visualized in a network constructed using Cytoscape software (version 3.6.02) and Gephi 0.9.2. The top interactions ($r \geq 0.6$ or $r \leq -0.6$) were prioritized to reduce network complexity and thereby allow key linkages to be appreciated. This “funneling” resulting in 24 DOM molecules and 16 OTUs with which to construct the network using Cytoscape. Gephi 0.9.2 was used to generate the separate network plots using the Force Atlas layout to connect DOM molecules and OTUs. Node sizes were correlated to the number of edges they contained, which resulted in larger nodes for OTUs compared to DOM molecules.

Results and Discussion

Soil chemical properties.

Long-term carbon-amendment or fertilizer application had a significant effect on chemical characteristics of soil (Figure S2). The contents of total carbon (TC) and DOC in sewage sludge (SS) and chicken manure (CM) treatments were higher than those in
chemical fertilizer (N) treatments and control (CK) (Figure S2a,b). Compared with 0.5N treatment (69.12 mg·kg\(^{-1}\)), 4SS and CM treatments increased DOC concentrations by 1.93 and 1.63 times, respectively (Figure S2b). High doses of organic carbon amendment (4SS and CM) increased TN and DTN significantly with the DTN increase by 2.4 times in 4SS treatment compared with 0.5N treatments (Figure S2c, d). 4SS and CM significantly decreased the soil C/N ratio \((P < 0.05)\) (Figure S2e). \(\text{NO}_3^-\)-N concentrations showed increasing trends with N application and organic carbon application, and was significantly higher (168 mg·kg\(^{-1}\)) in the 4SS treatments compared to the control (22.8 mg·kg\(^{-1}\)) (Figure S2f). \(\text{NH}_4^+\)-N concentrations across all treatments were not significantly different (Table S7). Soil pH ranged from 7.55 to 7.99 and no significant differences were observed among treatments (Table S7). Researches have demonstrated that sustained organic amendment could influence soil agroecosystem characters and usually resulted in higher nutrient contents compared with N-containing chemical fertilizer treatments \(^{11}\). Our results are consistent with previous researches that have demonstrated that long-term application of organic fertilizer influenced the organic matter content in soil and improved soil quality \(^{49,50}\).

**An overview of the variation and complexity of DOM composition.**

General characteristics of DOM revealed unique molecular composition harbored by different carbon-augmentation and fertilizer application practices (Figure 1, Table 1). A total of 6,428 molecular formulae were putatively assigned (average of all samples 3,607). Only 23.7% (1,521 molecules) of all molecular formulae were shared in all tested soil samples (Figure S8). Moiety molecular mass covered a range from approximately 132 to 599 Daltons. The DOM composition was comprised of heteroatomic compounds, such as CHO, CHON and CHOS (delineated by elemental formula combinations). CHON were most abundant (50.3% ~ 58.3% of all molecules).
followed by CHO (37.0% ~ 44.0%) and CHOS (3.3% ~ 8.7%) (Table 1).

The application of inorganic fertilizer (1N) increased the content of CHON by 6.0%, while the abundance of CHOS maintained at 3.3% with respect to the control (3.3%) (Table 1). It is likely that more N was available to microorganisms due to the N fertilization and more N containing organics were produced through microbial metabolism. Carbon amendment (SS and CM) increased the abundance of CHON and CHOS with the highest abundance of CHON in CM treatments and the highest abundance of CHOS in 4SS treatments (Table 1). The application of sewage sludge increased CHOS abundance by a factor of two to three (Table 1). The increase in S-bearing moieties is most likely related to the delivery of these molecules (and their metabolism post application) in SS and CM treatments. As sludge (SS) is acknowledged to be rich in sulfur (0.7 – 2.1%).

Different heteroatomic classes (i.e. Oₓ, NₓOᵧ, OₓSᵧ) in DOM were existed in all treatments. Compared to the inorganic fertilizer treatments, more types of heteroatomic classes were observed in the treatments with high dose carbon-augmentation (Table 1). Comparisons of DOM features in all treatments were based on H/C and O/C ratios, aromaticity index (AI), double bond equivalence (DBE) and H/C cutoffs. The relative abundances of DOM components were significantly different among treatments (Figure 1) (ANOSIM: R = 0.4872, P = 0.001). The relative abundance of each DOM component was significantly different among treatments as well (ANOVA, P < 0.05) (Table S6).

Lignin-like DOM compounds were dominant in all soil samples, accounting for 54~63% of all assigned molecules (Figure 1). The proportions of recalcitrant components (H/C < 1.5), such as lignin, condensed aromatics and tannins were higher than the proportions of labile components (H/C ≥ 1.5) in SS and CM treatments. The molecular composition in CM treatment covered much lower H/C and wider O/C ratio (ANOVA, p < 0.05)
(Table 1), indicating greater recalcitrance\textsuperscript{25}.

The evidence that carbon amendment practices (i.e. SS and CM) increased the stocks of recalcitrant organic carbon are significant, and of global importance. If rejuvenated soil carbon is labile then gains in soil carbon stocks will be transient (as augmented carbon is mineralized back into CO\textsubscript{2}). However, as our results indicate, SS and CM amendment (over a decadal period) resulted in an increase in recalcitrant carbon moieties. This evidence is salient to carbon sequestration and climate change mitigation as it highlights the potential for SS and CM carbon amendments to increase both the size of the soil carbon reservoir and the recalcitrance of this carbon. In relation to the 4\% Initiative \textsuperscript{54} our results affirm that soil carbon augmentation, with SS and CM, could make a pragmatic contribution to the 4\% aspiration.

Characterization of DOM chemodiversity.

The application of inorganic fertilizer and organic fertilizers changed the chemodiversity of DOM molecular composition in soil, with significant differences among treatments being reflected at a molecule level (Figure S3). The Chao 1 diversity significantly increased following the application of SS or CM (ANOVA, $P < 0.0001$, F value = 9.076). High dose application of organic fertilizer significantly increased DOM chemodiversity. CM application increased the Chao 1 diversity of DOM, although the increased level was less than those of high dose sewage sludge treatments. Non-metric multidimensional scaling analysis (NMDs), showed the DOM compositions in organic augmentation treatments to be markedly different from those of control and inorganic fertilizer treatments (stress = 0.05) (Figure 2). Additionally, Spearman rank order correlations revealed significant multicollinearity among Chao 1 index, soil pH, TC, TN, DOC, DTN, C/N ratio and NO\textsubscript{3}\textsuperscript{−} (Table S4). When soil chemical variables were fitted to the RDA plot (Figure S4a), DOM molecular composition was found to be
significantly correlated with DOC, DTN and NO$_3^-$ ($P = 0.001$). The variations of soil DOM chemodiversity under different fertilization significantly correlated with soil chemical factors, indicating that soil chemical factors that have distinct influence on soil BCC can also effect soil DOM molecular diversity.

Using the LEfSe analysis, a total of 651 DOM moieties (referred as chemo-markers) emerged to explain the greatest difference among treatments. By plotting these chemical markers, in a van Krevelen plot according to each moiety’s O/C ratio versus H/C ratio, observation of major groupings was simplified (Figure 3). Thus, a pronounced boundary, differentiating inorganic fertilizer treatments from organic augmentation treatments, was revealed. DOM molecules in the control were dominated by compounds belonging to the aliphatic category (H/C > 1.5). Similarly, chemo-marker molecules in inorganic fertilizer treatments were noted to be indicative of labile organic compounds (H/C > 1.5) i.e. proteins/amino sugars and carbohydrates. This result highlights long-term application of inorganic fertilizer in reducing the abundance of recalcitrant soil carbon moieties. Mulvaney reported that the amendment of inorganic fertilizer increased mineralization of native soil carbon and nitrogen.

In contrast, chemo-markers in SS and CM treatments were distinct from the chemo-markers in CK and N treatments (Figure 3), these moieties belonged primarily to recalcitrant compounds (H/C < 1.5). These, more recalcitrant compounds noted in the SS and CM treatments, belonged mainly to the category of humic-like compounds i.e. condensed aromatics, phenolic and highly unsaturated compounds and polyphenols. These results are consistent with previous reports that have indicated sludge amendments to increase humic matter contents in soils. Our results add new insight, in terms of the molecular fingerprint of the chemodiversity of DOM in soils augmented with organic carbon over a decadal period. These results are significant as they
highlight the benefits organic carbon augmentation can realize in terms of both building soil carbon stocks and increasing the recalcitrance of these carbon stocks. As highlighted above, these findings support the use of organic carbon amendments with potential to make long-term contributions to achieving 4‰ aspirations.

Characterization of bacterial community composition (BCC).

After assembling and quality filtering, 28,169 - 172,449 sequences were identified per sample (average of 80,361). These sequences were assigned into 7,872 OTUs at a 97% identity level. The most dominant phyla across all samples was the copiotrophic taxa, *Proteobacteria* (27.4% - 33.0%, Figure 4a). Other prevalent phyla across all treatments were, *Actinobacteria*, *Acidobacteria* and *Chloroflexi* (>10%) (Figure 4a). The similar trends in BCC have been reported in other long-term field experiment on fluvo-aquic soils. Various types of inorganic fertilizers and manure were fertilized in fluvo-aquic soil for 24 years. Soil BCC were all dominated by the *Proteobacteria*, *Acidobacteria*, and *Actinobacteria*. Soils amended with organic carbon and inorganic fertilizer had markedly, and significantly, different bacterial communities compared to each other and the control soil (ANOSIM, R = 0.7666, P = 0.001) (Figure 4). The relative abundance of *Acidobacteria* was lower in 4SS (14.2%) and CM (14.5%) compared to other treatments. The amendment of organic materials (CM & 4SS) increased the relative abundance of *Actinobacteria*, while decreased the relative abundance of *Chloroflexi*, especially at high doses of sludge via significant test (Figure 4a). These observations are consistent with previous studies in which organic matter amendment stimulated copiotrophic taxa (i.e. *Proteobacteria* and *Actinobacteria*) growth. *Proteobacteria* and *Actinobacteria* taxa have previously been reported to be dominant in soil under long-term organic carbon augmentation. In contrast, BCC was directed towards an increase in the oligotrophic taxa, *Acidobacteria* that was
present at significantly high frequencies in control, or N treatments. Proteobacteria have been putatively recognized as copiotrophic taxa (taxa that thrives in conditions of elevated C and N availability and exhibits relatively rapid growth rates) and favors nutrient-rich conditions and associated with carbon rich regimes. In contrast, Acidobacteria is considered to be an oligotrophic taxon that exhibits relatively slow growth rate and ability to metabolize nutrient-poor substrates. The relative increase (25.5%) in Acidobacteria increased reported here is consistent with the BCC shifts under twenty-three years of nitrogen-containing inorganic fertilization applications.

Further comparison of the BCC at the class level revealed, Actinobacteria as the dominant group (17.4%), closely followed by Acidobacteria (16.1%) and Alphaproteobacteria (11.8%) (Figure 4b). Another highly abundant class was Gammaproteobacteria (6.05%). Actinobacteria was the most abundant class in samples of 4SS, while Acidobacteria was dominant in 0.5SS, 2SS. In the control (CK), 0.5N, 1N, 1SS and CM Alphaproteobacteria was the most abundant class. The relative abundance of Actinobacteria increased in SS and CM treatments with Actinobacteria dominating in 4SS at the class level.

The overall pattern of BCC in the NMDS plot (Figure S5) (stress = 0.05) suggested that BCC was altered by organic carbon amendments, and BCC in SS and CM group were markedly different from BCC in 0.5N and 1N group. Alpha-diversity of bacteria was increased in SS and CM treatments (especially at high SS doses) (Table S3). A redundancy analysis elucidated the relationships between the BCC and soil factors in the different fertilization treatments. The RDA ordination plot (Figure S4b) indicated that soil DTN, DOC and NO$_3^-$ were the most important variables that influencing BCC.

Previous studies have indicated that organic carbon and inorganic fertilizer application have profound shaping influence on soil microbial community structure,
with implications for the cycling of carbon, the regulation of soil ecosystem services, such as nutrient flows and greenhouse gas emission. The observed shifts in BCC are suggested to be in response to decreasing soil fertility associated with long-term chemical fertilizer application that reduced the nutrient availability and increases nutrient loss. Correspondingly, the low-fertility soils supported oligotrophic ecosystems, whereas high-fertility soils support eutrophic ecosystems. In contrast, long-term organic carbon-augmentation improved DOM quality, and promoted the growth of copiotrophic taxa. Thus, our results suggested that organic carbon-augmentation supported a bacterial community shift to one indicative of a eutrophic ecosystem.

**Linkages between soil bacterial communities, DOM molecular composition and soil properties.**

The interconnections between chemodiversity of DOM and BCC were explored using co-occurrence network analysis. Results revealed strong and significant associations between DOM molecules and specific taxa (Figure 5). The network pattern indicated taxa of the same phyla or same class had diverse associations with DOM of contrasting chemical characteristics, i.e. having opposite correlations to the same category of DOM compounds or having correlations to the molecules belonging to distinct regions of chemical composition. These results reveal evidence that *Nitrospira* specialized on typical DOM molecules which might be utilized for defining the possible ecological niche.

There were 52,319 pairs of correlations, and 7,944 strong correlations (|R| > 0.9) between the DOM molecule dataset and the OTUs dataset (Figure S6). The top 100 OTUs with the highest relative abundance and the top 100 most abundant DOM molecules were considered for network analysis (Figure 5, Table S5). A total number
of 1,105 pairs of correlations were established among these top DOM molecules, OTUs and environmental factors. At $R \geq 0.6$, 135 correlations persisted, of which 40 linked 26 DOM molecules and 17 OTUs. All 17 OTUs belonged to the taxa *Acidobacteria* (7), *Proteobacteria* (3), *Actinobacteria* (3), *Nitrospirae* (2), *Chloroflexi* (1) and *Planctomycetes* (1), respectively. Sixteen DOM molecules belonged to recalcitrant compounds ($\text{H/C } < 1.5$). *Proteobacteria*, in particular, showed strong positive correlation (red lines) with recalcitrant compounds ($\text{H/C } < 1.5$) and negative correlation (black lines) with aliphatics ($\text{H/C } > 1.5$) (Figure 5), these chemical traits being consistent with high-dose SS and CM treatments (Figure 3). *Acidobacteria* showed negative correlations with recalcitrant compounds in all treatments (Figure 3). These observations support the hypothesis that infertile soil with low nutrient availability (consistent with CK and 0.5N treatments) generally selected for *Acidobacteria* $^{12, 34}$. A group of ten labile DOM molecules belonged to aliphatic ($1.5 < \text{H/C } \leq 2.0$) and carbohydrate ($0.6 < \text{O/C } < 1.2$ and $1.5 < \text{H/C } < 2.2$). A group of distinct DOM molecules showed strong correlations with more than one OTU (these belonging to seven different phyla) whereas the remaining DOM molecules were correlated with either, *Acidobacteria*, *Nitrospira*, or *Proteobacteria*. Strongly restricted correlations were observed between *Proteobacteria* and aliphatic-like compound, whereas *Acidobacteria* and *Nitrospira* had strong correlations that were almost exclusively with highly unsaturated hydrocarbons, phenolic compounds and lignins. In addition, *Nitrospira* showed strong negative correlations to the recalcitrant compounds, and *Desulfurellaceae* showed strong negative correlations to the CHOS class (especially $\text{C}_9\text{H}_{18}\text{O}_6\text{S}_1$, Figure 5). This may suggest that *Nitrospira* and *Desulfurellaceae* specialized on specific DOM categories that are not intensively utilize by other taxa. *Desulfurellaceae* is common sulfate-reducing bacteria in sludge and is capable of
consuming CHOS compounds (these noted to be abundant in SS and CM treatments (Table 1)). The co-occurrence in network supports the findings of specialization on specific substrate. The separate network plots showed significant differences between the inorganic fertilizer treatments and organic carbon treatments (Figure 6). In the CK and 0.5N treatment, the network had 350 and 278 edges, and 4SS and CM had 537 and 401 edges, respectively. In addition, the more active nodes were detected in the organic carbon-amendments treatments than in the inorganic fertilizer treatments. The node having more than 7 edges was defined as the network hub, which was active in mediating interactions. The organic carbon-amendments (especially the high dose treatments) maintain a more complex network structure.

Previous research regarding linkages between DOM and BCC has only focused on the effects of the content of soil carbon, the appreciation of their relationships with each other is strikingly limited on the broad molecular level due to the complexity in composition of both. Our results are significant as they reveal, in unprecedented detail, associations between DOM chemodiversity and BCC diversity. Soil bacteria-DOM interaction was demonstrated in strong correlations between specific bacterial taxa and particular DOM molecules, thus, suggesting bacterial specialization on particular substrates. The number of active hubs in the carbon-amendments treatment was more than in the inorganic fertilizer treatments, indicating that a greater diversity of soil bacteria interacted with a greater diversity of DOM molecules in treatments subjected to protracted organic carbon amendment. To the best of our knowledge, this is the first report the co-variation of soil DOM composition and BCC.

Taking advantage of technological advances in analytical chemistry, molecular biology and informatics, we explored how the BCC and DOM chemodiversity were altered (in long term, decadal, field experiments) in response to carbon-amendment and
application of inorganic fertilizers. This research highlights the manifold associations between the diversity of microbiota and the heterogeneity of soil DOM under long-term organic carbon amendment and inorganic fertilization practices. Our results bring new insight to the negative impacts of protracted inorganic fertilizer application on the DOM resource in soil and the BCC it supports. Our results indicate that protracted organic carbon amendments not only increased soil carbon stocks but also their recalcitrance. In addition, SS and CM amendments shaped BCC to an indicative state of improved soil health and one that has the potential to improve delivery of soil ecosystem service delivery within agroecosystems. These two lines of evidence affirm that soil carbon augmentation, with SS and CM, could make a pragmatic contribution to the 4‰ aspiration to (re)build resilient soil carbon stocks while improving soil health and the delivery of beneficial soil ecosystem services. Our results might contribute to the defining of a mechanism to translate current scientific knowledge, regarding soil carbon status, into actionable pathways that might inform new agricultural or land use policy to bring to fruition the 4‰ vision.

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Notes

The authors declare no conflict of interest.
Supporting Information

Details on supportive methods and discussion, additional details of the field layout and experiment (Figure S1, Table S1 and Table S2); soil chemical variables (Figure S2), diversity index of soil DOM and bacterial community (Figure S3 and Table S3), Redundancy analysis (RDA) of DOM molecular composition and bacterial community composition (Figure S4), NMDS analysis of bacterial community composition (Figure S5), Co-occurrence network visualizing the DOM-Bacteria interactions (Figure S6).

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Table 1 FT-ICR MS characteristics of DOM composition.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CHO (%)</th>
<th>CHON (%)</th>
<th>CHOS (%)</th>
<th>Number of Heteroatomic class</th>
<th>C</th>
<th>H</th>
<th>O</th>
<th>MW</th>
<th>DBE</th>
<th>H/C ratio</th>
<th>O/C ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>44.0a</td>
<td>52.0a</td>
<td>3.33a</td>
<td>49a</td>
<td>15.95ab</td>
<td>17.61d</td>
<td>7.00a</td>
<td>334.66a</td>
<td>8.09a</td>
<td>1.10c</td>
<td>0.44a</td>
</tr>
<tr>
<td>0.5N</td>
<td>43.3ab</td>
<td>53.0a</td>
<td>3.33a</td>
<td>52b</td>
<td>15.92ab</td>
<td>16.49bc</td>
<td>7.12a</td>
<td>335.40a</td>
<td>8.63b</td>
<td>1.04b</td>
<td>0.45ab</td>
</tr>
<tr>
<td>1N</td>
<td>39.0b</td>
<td>58.0b</td>
<td>3.33a</td>
<td>55c</td>
<td>16.12b</td>
<td>16.33b</td>
<td>7.54b</td>
<td>345.77bc</td>
<td>8.96d</td>
<td>1.01b</td>
<td>0.47c</td>
</tr>
<tr>
<td>0.5SS</td>
<td>43.3b</td>
<td>50.3a</td>
<td>6.67b</td>
<td>51b</td>
<td>16.32c</td>
<td>17.73d</td>
<td>7.18a</td>
<td>342.76b</td>
<td>8.39b</td>
<td>1.09c</td>
<td>0.44a</td>
</tr>
<tr>
<td>1SS</td>
<td>38.7b</td>
<td>53.7c</td>
<td>7.67c</td>
<td>56c</td>
<td>16.27cd</td>
<td>16.88bc</td>
<td>7.51b</td>
<td>348.41cd</td>
<td>8.82cd</td>
<td>1.04b</td>
<td>0.46c</td>
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<tr>
<td>2SS</td>
<td>37.7b</td>
<td>55.3c</td>
<td>7.00c</td>
<td>56c</td>
<td>16.33cd</td>
<td>16.73bc</td>
<td>7.62b</td>
<td>351.03d</td>
<td>8.97d</td>
<td>1.02ab</td>
<td>0.47c</td>
</tr>
<tr>
<td>4SS</td>
<td>37.0b</td>
<td>54.3c</td>
<td>8.67c</td>
<td>56cd</td>
<td>16.46d</td>
<td>17.09d</td>
<td>7.57b</td>
<td>352.60d</td>
<td>8.91cd</td>
<td>1.04b</td>
<td>0.46bc</td>
</tr>
<tr>
<td>CM</td>
<td>36.7ab</td>
<td>58.3b</td>
<td>5.33b</td>
<td>57d</td>
<td>15.87a</td>
<td>15.71a</td>
<td>7.66b</td>
<td>345.34cd</td>
<td>9.04d</td>
<td>0.99a</td>
<td>0.48d</td>
</tr>
</tbody>
</table>

MW - molecular weight, DBE - double bond equivalent. Significance level: *P < 0.05.
Figure legends

**Figure 1** van Krevelen diagram-derived relative abundance (%) of classification classes of the DOM components (from FT-ICR MS analysis) in: control soil (CK), inorganic fertilizer treatments (0.5N and 1N), sewage sludge treatments (0.5SS, 1SS, 2SS and 4SS) and chicken manure treatments (CM).

**Figure 2** Distribution patterns of DOM molecules composition. Non-metric Multidimensional scaling (NMDS) analysis of DOM molecules based on Bray-Curtis distance.

**Figure 3** Linear discriminant effect size analysis (LEfSe) of DOM chemo-marker molecules that are enriched in different treatments. Number of chemo-marker molecules: 651 in total; 54 in sludge; 293 in manure; 47 in N-chemical fertilizer, and; 257 in the control. Molecule compounds with no significant differences are not shown.

**Figure 4** (a) Relative abundance of bacteria community composition components at the phylum level. (b) Relative abundances of bacteria community composition at class level. “Others” include low abundance (< 1%) bacteria and the taxonomically unassigned sequences at class level.

**Figure 5** Interaction network analysis of top 100 most abundant bacterial OTUs and top 100 most abundant DOM molecules that were significantly correlated (P < 0.05, |R| > 0.6). Circles, DOM molecules; Triangles, Bacterial OTUs (green); DOM molecules relative abundances are set proportional to node size. Nodes are colored according to DOM category, e.g. aliphatic compounds (light blue) and recalcitrant compounds (red). Positive correlations are indicated using red lines, negative correlations are indicated using black lines.
Figure 6 Statistically significant and strong co-occurrence relationships between DOM molecules and bacterial OTUs within different treatments. Network plots for (a) CK, (b) 0.5N, (c) 4SS and (d) CM treatments. Nodes represent DOM molecules and OTUs with significant relationships. The color of each node indicates the OTUs from different phylum and labile (light blue) or recalcitrant (dark blue) DOM molecules (see key). The size of the nodes (circles) is proportioned to the number of the connections (degree).
Figure 1

The bar chart illustrates the relative abundance (%) of various compounds across different treatments: CK, 0.5N, 1N, 0.5SS, 1SS, 2SS, 4SS, and CM. The compounds are categorized into:

- **Protein/amino sugars**
- **Lipids**
- **Lignins**
- **Condensed aromatics**
- **Carbohydrates**
- **Tannins**
- **Unsaturated hydrocarbons**

Each bar represents a specific treatment, and the length of each section within the bars indicates the percentage contribution of each compound.
Figure 3

**Figure Description:**

A scatter plot with a 2D Cartesian coordinate system where the x-axis represents the O/C ratio, and the y-axis represents the H/C ratio. The plot contains data points grouped by different categories:

- **Green circles (CK):** Represent aliphatic and highly unsaturated compounds.
- **Blue circles (N):** Represent vascular plant-derived polyphenols.
- **Red circles (SS):** Represent combustion-derived polycyclic aromatics.
- **Orange circles (CM):** Represent highly unsaturated and phenolic compounds.

The plot highlights the distribution of different compounds across the O/C and H/C ratio ranges. The labels indicate the types of compounds, with specific categories highlighted for clarity.
Figure 6

(a) CK  (b) 0.5N  (c) 4SS  (d) CM