Early-stage effects of carbon-rich soil amendments stimulate retention-related nitrogen genes while maintaining nitrogen and yield levels

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ABSTRACT

Understanding the effects of soil amendments and low disturbance practices on soil health, nutrient cycling and microbial activity is essential for improving agricultural sustainability. Ramial chipped wood (RCW) is a promising carbon-rich organic soil amendment but its effects on microbial activity,

nitrogen (N) cycling genes and microbial taxa, particularly across soil depth, remain poorly understood. This study aimed to evaluate the short-term effects of RCW applications following a no-till practice on various soil properties including microbial composition and N cycling genes, during the second year after RCW incorporation. The experiment was conducted using tomato (*Solanum lycopersicum*) as a crop species in the Mediterranean region. We compared the surface (0-20 cm) and subsurface (20-25 cm) metagenomes of RCW-treated soils with those treated with standard N-rich organic pellet, as a control, (CTL) and compost (CMP).

RCW, particularly at high doses (RCW-HD), increased soil organic carbon and microbial biomass at an early stage. Despite a 50% reduction in organic fertiliser use, RCW-HD did not reduce N availability and crop productivity, suggesting improved N use efficiency. Several N-cycling gene abundances were elevated under CTL compared to RCW-HD, including the nitrification-related *pmoA-amoA* (+42%) and *pmoC-amoC* (+72%), and the denitrification-related *nosZ* (+14%). The RCW-HD no-till system increased nitrate reduction assimilation (+13% *nrtABC*) and favoured N-fixing bacterial genera such as *Terrihabitans, Ferriphaselus, Azospira* and *Rhodopseudomonas*. Soil depth significantly influenced 72% of the N-cycling genes, with key genes being more abundant at the surface. These results highlight the potential of RCW to improve N retention and soil fertility, while reducing fertiliser dependence and greenhouse gas emissions. They also support sustainable practices in regenerative agriculture by highlighting how microbiomes contribute to the efficiency of nitrogen cycling.

Keywords: Shotgun metagenomics; Biogeochemical process; Microbial activity; Organic agriculture; Soil organic matter; Soil regeneration.

1. Introduction

Soil degradation is a global environmental challenge that threatens agricultural productivity and food security, with over 30% of soils classified as degraded, reducing their ability to support crops and

47 sequester carbon (C) (Vasu et al., 2024). Agricultural intensification exacerbates this problem by
48 depleting soil organic carbon (SOC), degrading physical properties, and reducing soil biodiversity, all
49 of which affect the sustainability of food production (Celik et al., 2010; Gogoi et al., 2021; Khasi et al.,
50 2024; Mamabolo et al., 2024). Soil restoration, i.e., increase of soil organic matter (SOM), is essential
51 to reverse these effects and improve soil health (Briedis et al., 2018). The use of organic amendments
52 such as compost, manure, biochar and ramial chipped wood (RCW) has shown promise in enhancing
53 SOM, microbial activity, and nutrient cycling, particularly nitrogen (N) mineralisation and organic
54 matter decomposition, ultimately improving soil fertility (Lemieux and Germain, 2001; Tahboub et al.,
55 2008; Gholami et al., 2016; Liu et al., 2024). RCW, with its high C:N ratio (often >60), slower
56 biodegradability, and lignin-rich composition, is characterized by its ability to promote microbial
57 activity, increase soil aggregation, and support long-term restoration (Daassi et al., 2020; Daassi et al.,
58 2024). The lignin content of RCW contributes to C storage, while the biodegradation of lignocellulosic
59 materials facilitates the release of nutrients, particularly N, which is essential for plant nutrition
60 (Daassi et al., 2020). In contrast, compost is produced through active decomposition processes and
61 generally has a lower C:N ratio (typically <20), reduced lignin content, and a higher concentration of
62 readily available nutrients (De Corato, 2020). Therefore, RCW offers distinct advantages in no-till
63 systems by contributing to long-term organic matter stabilisation and enhancing microbial habitat
64 structure, complementing the slow mineralisation dynamics of minimally disturbed soils and
65 improving nutrient retention over time. To the best of our knowledge, no previous studies have
66 investigated the effects of RCW application within organic no-till systems on soil N processes. In no-
67 till systems, the slow decomposition of RCW supports the development of microbial communities that
68 enhance N retention and mineralisation, particularly under low-disturbance practices. In addition,
69 previous studies have reported that low-disturbance practices such as reduced tillage (Y. Li et al.,
70 2020), no-tillage (Gao et al., 2016) and stover mulching (Qin et al., 2021) can restore soil health by
71 promoting SOC storage, improving nutrient and water use efficiency (Jat et al., 2020), reducing

nutrient losses (Seitz et al., 2019). These practices can be further enhanced when combined with diverse crop rotations (Pittelkow et al., 2015).

The soil regeneration process involves biochemical changes driven by the intrinsic interactions between microorganisms, plant roots and soil (Chen et al., 2024). These changes typically occur in the first few centimeters of soil, especially under no-till management, due to the concentration of organic amendments and rooting patterns in the surface layer (Deng et al., 2022; Li et al., 2024). Microbial activity plays a critical role in driving C and N cycling, which is influenced, among others by the availability of organic compounds such as lignin and cellulose that provide energy for microbes (German et al., 2011; Tian et al., 2020). These processes influence soil structure and SOM dynamics, including SOC accumulation and decomposition, which in turn leads to plant nutrient mineralisation (Daly et al., 2021; Li et al., 2023). In addition, free-living bacteria such as *Rhizobium* (Janczarek et al., 2024) and *Azospirillum* (Fukami et al., 2018) enhance soil productivity by contributing to plant nutrition alongside external N inputs (Oldroyd et al., 2011; Janczarek et al., 2024). Thus, soil microorganisms as decomposers and divers of biogeochemical cycles are essential for SOM dynamics and nutrient cycling, play a key role in soil C and N cycling and enhance soil fertility and C storage potential (Daly et al., 2021; Wang et al., 2023).

Assessing the short-term responses of soil microbial populations to complex organic amendments in minimally disturbed soils is essential for understanding the role of microbes in biogeochemical cycles. Most biogeochemical transformations occur during short periods of enhanced microbial activity (Chuckran et al., 2021). This stimulation often results from localized increases in nutrient concentrations, such as those found in the rhizosphere or areas undergoing decomposition of fresh organic matter (Chuckran et al., 2021). The hypothesis of this work was that the incorporation of large amounts of RCW into the topsoil, combined with no-tillage practices, will increase C availability to promote N-cycling microbial processes and build up N reserves under reduced fertiliser regimes.

Compost application represents a middle ground between C-rich (i.e., RCW) and N-rich (i.e., organic pellet) applications. Although nutrient availability in compost is higher than in RCW, most of the plant material has been largely broken down prior to application (De Corato, 2020). The pre-decomposition limits the stimulation of microbial activity as the substrate is less bioavailable, and the microbial communities introduced via compost are unlikely to strongly influence the native soil microbiome (Xu et al., 2023). Consequently, compost applications are expected to have weaker effects on microbial activity and SOM dynamics compared to fresh organic amendments. The aim of this study was to analyse soil chemical, biochemical and metagenomic properties to understand N cycling processes during short-term soil regeneration after C-rich RCW application under no-tillage practice. In this study, we compared the use of RCW as an organic amendment in a no-tillage management system with two conventional fertilisation practices: plant-based compost (characterised by higher nutrient availability due to pre-decomposition) and granulated organic N fertiliser (characterised by slow-release N content). Both conventional systems were applied under conventional tillage. This approach should provide valuable insights for optimizing organic regenerative farming practices by balancing nutrient availability, microbial stimulation, and maintenance of soil structure.

2. Materials and methods

2.1. Site description and experimental design

The study was conducted at Cal Notari, a commercial horticultural farm in Sant Boi de Llobregat, Barcelona, Spain (41°19’4.8’’ N, 2°3’3.6’’ E). The soil is classified as silty clay loam (sand/clay ratio 5.5:28.5) with 36.17% calcium carbonate and an alkaline pH (8.6). The region has a Mediterranean climate. During the study period, the following average temperatures were recorded May 2021 (start of experiment) 17.0 ± 0.4 °C, summer 2021 24.0 ± 2.5 °C, autumn 2021 17.2 ± 1.8 °C, winter 2021-2022 9.6 ± 1.0 °C, spring 2022 15.2 ± 1.6 °C and summer 2022 25.7 ± 2.7 °C. The corresponding

cumulative precipitation was 32, 23, 179, 21, 138 and 92 mm, respectively. Meteorological data were obtained from the Servei Meterològic de Catalunya (https://meteo.cat/). Before the experiment began, baseline soil properties were measured (**Table S1 in Supplementary data**).

In May 2021, four organic management systems were established in a randomized block design (four replications) (**Fig. 1**). The treatments included: (1) untreated control (CTL): no amendments or fertiliser were applied; (2) compost treatment (CMP): 1.28 kg/m^2 of woody plant residue compost; (3) low-dose ramial chipped wood (RCW-LD): 7.5 kg/m^2 of RCW; (4) high-dose RCW (RCW-HD): 15 kg/m^2 of RCW. The RCW is derived from C-rich pruning residues from peri-urban areas within the municipality of Sant Boi de Llobregat. Organic amendments were incorporated into the top 20 cm using a 'Rotovator' milling machine. The 16 plots (1.5x7.5 m) were separated by 1 m and arranged in two 2 m wide paths. The soil surface was covered with biodegradable plastic and a drip irrigation system was installed.

The experiment followed a crop rotation: sweet potato (*Ipomoea batatas* var *Beauregard*), followed by spinach (*Spinacia oleracea*) and fava bean (*Vicia faba*). No fertiliser was applied between May 2021 and April 2022. Before tomato planting (May 2022), fertilisation varied between treatments: CTL received 0.21 kg/m^2 of commercial N-rich organic pellet fertiliser (Labinor N-10) applied by rotary tillage, CMP received 1.28 kg/m^2 of woody plant residue compost applied by rotary tillage, while RCW-LD and RCW-HD received 0.12 kg/m^2 of N-rich organic pellet applied as surface mulch.

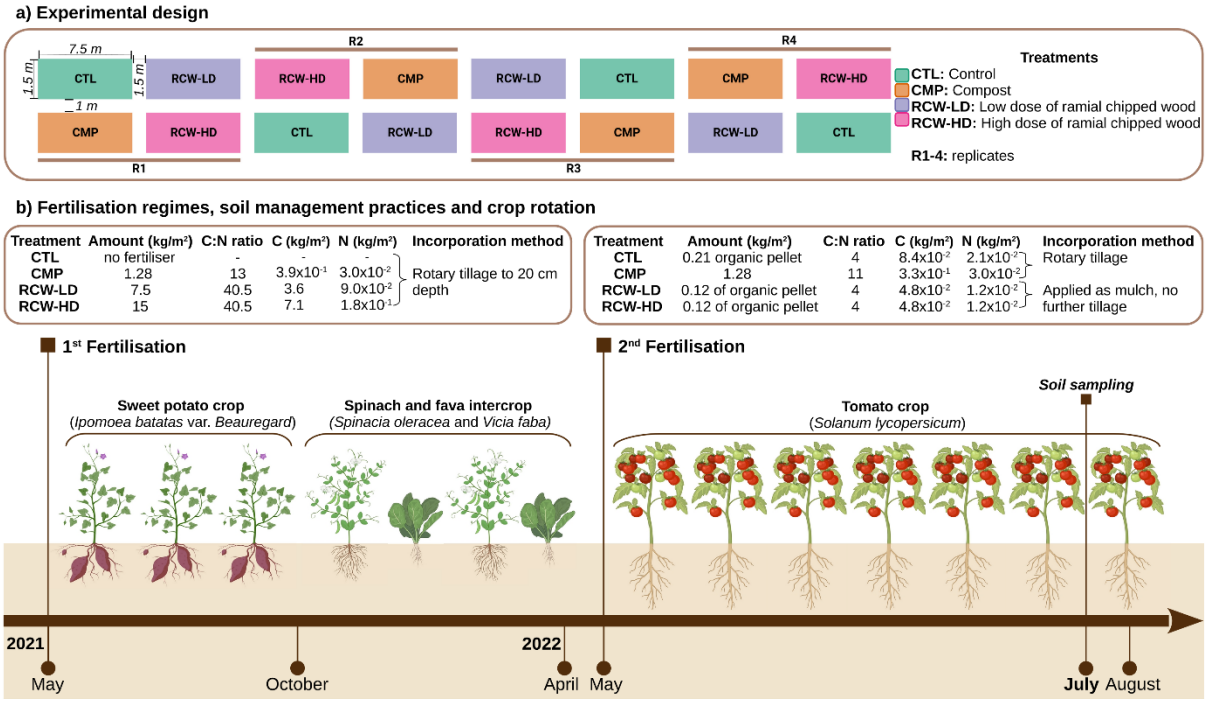

Fig. 1. Overview of the experimental design and fertilisation schedule for the soil management treatments. (a) Schematic representation of the experimental design showing the treatments and their representative replicates. **(b)** Description of fertilisation regimes, soil management practices and crop rotation sequence. The crop rotation timeline shows the months and years of cultivation, with two different fertilisation events highlighted. Each fertilisation event is accompanied by a table detailing the amount of fertiliser applied, including C/N ratio and C and N inputs, and the method of incorporation (tillage vs. no-tillage). Soil sampling for this study was carried out in July 2022, during the tomato growing season.

2.2. Soil sampling and chemical analysis

Soil samples were collected in July 2022, 14 months after RCW was incorporated. Eight soil subsamples (5 cm in diameter) were collected from 0-20 cm and from 20-25 cm depth in each plot, designated as surface and subsurface soil, respectively. Samples were collected for each treatment, before tomato maturity and close to the tomato plants. A total of 32 samples were collected, consisting of 16 samples from the 0-20 cm depth and 16 from the 20-25 cm depth, representing four replicates for each treatment at each depth.

To assess microbial metabolic activity and capacity for organic matter decomposition, respiration demand was measured over a 17-day incubation period according to Jenkinson & Powlson (1976). Prior to incubation, 20 g soil samples were moistened to 60% field capacity with distilled water and incubated in the dark at room temperature. Samples were placed in airtight containers containing 20 mL of 0.1 M NaOH and conductivity (µs/cm) was measured every 48 h. K_2SO_4 extractions were performed before and after incubation. Microbial biomass C and N were determined by fumigation extraction (Vance et al., 1987) using ethanol-free $CHCl_3$ for 24 h fumigation followed by extraction with 0.5 M K_2SO_4. The microbial C/N ratio was analysed accordingly. Microbial biomass C was calculated as the difference in soluble organic C before and after incubation, while the metabolic quotient (qCO_2) was determined as the ratio of microbial respiration (µg C-CO_2) to microbial biomass increment (µg C/g) over 17 days. Nitrate (NO_3^-) (Cataldo et al., 1975) and ammonium (NH_4^+) (Sims et al., 1995) concentrations were measured by colorimetric methods from 0.5 M K_2SO_4 extracts, with mineral N calculated as their sum. Soluble organic C was determined using the EPA 9060 method with acidification (6% HCl, pH=2) and analysis by non-dispersive infrared gas spectrometry (NDIR) (Analytik Jena, model multi-N/C 3100, Thuringia, Germany).

Crushed soil samples were used to measure SOC and total N. SOC was quantified by dichromate oxidation (Moebius, 1960), while total N was measured by using an isotope ratio mass spectrometer (CF IRMS, Flash 2000 HT, Thermo Fisher Scientific). Substrate induced respiration was assessed using the MicroResp™ technique (Llimós et al., 2021) to evaluate the metabolic response of soil microbiota to different C sources. The C sources were selected based on their potential to influence the metabolic processes of soil microbiota, and they are listed in **Table S2** in the Supplementary Information.

2.3. Soil metagenomic extraction and sequencing

DNA soil samples were extracted using the E.Z.N.A.® soil DNA extraction kit (Omega Bio-Tek, USA), according to the manufacturer's instructions. The quality and quantity of the DNA samples were checked using a NanoPhotometer P-class (Implen GmbH, Germany). Shotgun metagenome sequencing was performed at Macrogen (Korea). Sequencing libraries were prepared directly from the extracted DNA using the Nextera XT DNA library preparation kit (Illumina). Finally, paired-end sequencing (150 bp read length) was performed on an Illumina NovaSeq6000.

2.4. Metagenomic data processing

Trimmomatic-0.39 was used for adapter removal (Bolger et al., 2014) and quality control of all raw sequences was checked using the FastQC-0.12.1 tool (Andrews, 2010). Taxonomic profiles were generated using the Kraken2 (v2-2.1.3) tool (Wood et al., 2019) based on *K*-mer composition. Raw reads were annotated against the RefSeq database https://benlangmead.github.io/aws-indexes/k2 (PlusPF, june 2023) for Bacteria, Archaea and Fungi profiling. The Kraken2 system labels the sequence with the lowest common ancestor (LCA) of all species sharing identical regions between two or more genomes. Abundance of the taxa was estimated using Bracken (Lu et al., 2017) at 98% accuracy. The combination of Kraken2 and Bracken allows the taxonomic assignment of the soil microbiome to be estimated with superior precision, sensitivity, F1 score and overall sequence classification (Edwin et al., 2024). For functional assignment, paired end reads from all samples were concatenated and aligned against the NCBI non-redundant protein database using DIAMOND (v.2.0.4) (Bağcı et al., 2021; Buchfink et al., 2021). Functional annotation was performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database and based on the KEGG orthology (KO) results (https://www.genome.jp/kegg/). N-cycle genes were selected for abundance calculation.

2.5. Statistical analysis and visualization

All statistical analyses and graphical visualisation were conducted using R (v.4.4.0) and R Studio (v.2024.04.1.+748) (R Core Team, 2024). The effects of experimental factors on soil chemical and biological properties and N cycling genes were analysed using linear mixed effects models (LME). For functional profiles, all gene data were first normalised using Microbiome analyst (Dhariwal et al., 2017). LME was performed using the *lme* function from the *nlme* package (v. 3.1.166) (Pinheiro et al., 2024). The model included organic management systems (CTL, CMP, RCW-LD, and RCW-HD), soil layer (0-20 and 20-25 cm of depth) and their interaction with block as a random effect. Statistical significance was assessed using the *anova* function, and multiple comparisons were performed using the *emmeans* function (EMMEANS package (Lenth, 2024)). Data transformations, including square root transformation, inverse square root, square root of variable plus one, logarithmic transformation and power transformation (squaring and cubing of data), were applied to meet model assumptions.

Taxonomic data were analysed using the *microeco* package (v.1.7.1) (Liu et al., 2021). After filtering and normalization of the data, we analysed 9236 Bacteria, 431 Archaea and 88 Fungi. Alpha diversity indices (Chao1, ACE, Shannon) were calculated using the *cal_alphadiv* function. In generalised lineal mixed models (GLMM), factorial effects were assessed with the *cal_diff* function. Beta diversity was calculated with the *cal_betadiv* function and Multivariate Analysis of Variance (PERMANOVA) was performed with 999 permutations (*cal_manova*). Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance visualised microbial community distribution.

N-cycle related taxa were identified from the KEGG pathway database (https://www.kegg.jp) and 1318 bacterial, 51 archaeal and 38 fungal taxa were analysed. The *cal_abund* function calculated relative abundances, while differential abundances were assessed using GLMM models (*trans_diff),* with FDR adjustments for P-*values*. Pearson correlations linked changes in microbial communities and N-cycle genes to soil properties, visualised via heatmaps (*pheatmap* package (Kolde, 2019)). Barplot with error bars was generated using *ggplot2* (Wickham, 2016) and *Rmisc* packages (Hope, 2022).

223 Redundancy analysis (RDA) was performed using the *vegan* package (v.2.6-6.1) (Oksanen et al.,
224 2024)to relate gene data to soil properties. Gene counts were Hellinger transformed using the
225 *decostand* function, while soil variables were using standarize method. Model selection used the *step*
226 function, with variance inflation factor (VIF < 3) assessed using *vif.cca* and adjusted R^2 calculated using
227 *RsquareAdj*. Permutation-based ANOVA tests (*anova*, 999 permutations) determined the significance
228 of the overall model, axes and variables. RDA biplots with linear scaling (scaling = 2) were used to
229 visualise the results. Two PERMANOVA models were performed using Bray-Curtis dissimilarities
230 (a*donis* function, *vegan* package).

231

232 **3. Results**

233 *3.1. Ramial chipped wood increases SOC and microbial biomass*

234 The application of RCW did not negatively affect tomato production, which was similar between
235 treatments as shown **in Table 1.** Despite of high variability observed in yield data of this commercial
236 on-farm experiment with a relative standard error (RSE) of 66% for the yield data, we observed mostly
237 lower RSEs for the main biochemical soil properties ranging between 4.6-27% and 4.7-47% for surface
238 and subsurface soil, respectively.

239 The application of high doses of RCW significantly increased SOC compared to the CTL treatment (P <
240 .05), as shown in **Table 1**. SOC concentrations were significantly higher on the surface than in the
241 subsurface layers, showing an increase of 76% (P < .001). Soluble organic C was significantly higher in
242 surface soils under the CMP treatment compared to the CTL treatment (P < .01), while it was
243 significantly lower in subsurface soils (P < .001). Total N concentrations were 95% higher in surface
244 soils than in subsurface soils (P < .001). Soil NO_3^- concentrations also increase significantly higher in
245 surface soil, with an increase of 287% (P < .001). Similarly, mineral N concentrations were 143% higher

in surface soils compared to subsurface soils ($P < .001$). In contrast, soil NH_4^+ concentrations showed a significant increase in subsurface soils with a 242% higher concentration compared to surface soils.

The change in soluble organic C (Δ soluble organic C) showed significant treatment effects, with CMP resulting in the highest uptake compared to other treatments at the surface ($P < .05$). Depth also significantly influenced Δ soluble organic C, with surface soils showing net uptake and subsurface soils showing a trend towards accumulation, with an increase of 152%. The incorporation of RCW significantly increased the microbial biomass of N compared to CMP ($P < .05$). The microbial biomass N increment (Δ microbial biomass N) showed significant treatment effects ($P < .05$) and depth effects ($P < .05$). At the surface, CMP had the highest concentration, in contrast, at subsurface depths, Δ microbial biomass N were generally lower. Microbial biomass C significantly increased by 23% in RCW-HD compared to CTL treatment in the surface layer ($P < .05$). The changes in microbial biomass C (Δ microbial biomass C) showed different responses to treatments and depth during incubation. At the surface, CMP showed the least reduction in microbial biomass C, significantly different from RCW-LD and RCW-HD, as indicated by statistical differences ($P < .05$). At subsurface depths, all treatments showed a consistent reduction in the changes of microbial biomass C. No significant differences in the metabolic coefficient were observed between treatments or soil layers. However, respiration showed a clear effect in the soil layer depth, with higher daily respiration rates at the surface ($P < .01$).

Table 1. Tomato crop production and soil chemical and biochemical properties under different organic management systems.

	Depth	Treatment								LME (*P-value*)		
Parameter	**(cm)**	**CTL**		**CMP**		**RCW-LD**		**RCW-HD**		**T**	**D**	**TxD**
Production (t/ha)	-	19.9 ± 12	A	15.1 ± 9.2	A	13.4 ± 10	A	17.4 ± 12	A	**.044.**	NA	NA
Soil chemical properties												
Total N (%)	0-20	0.203 ± 0.03		0.232 ± 0.01		0.208 ± 0.01		0.195 ± 0.02		.207	**<.001 *****	.559
	20-25	0.104 ± 0.01		0.111 ± 0.01		0.104 ± 0.01		0.110 ± 0.00				
SOC (%)	0-20	2.53 ± 0.11	B	2.88 ± 0.22	AB	2.90 ± 0.12	AB	3.05 ± 0.21	A	**.031 ***	**<.001 *****	.195
	20-25	1.63 ± 0.16		1.55 ± 0.21		1.66 ± 0.23		1.62 ± 0.11				
NO_3^-(μg/g)	0-20	58.3 ± 7.5		61.1 ± 6.6		59.5 ± 8.2		51.4 ± 1.6		.768	**<.001 *****	.874
	20-25	16.3 ± 2.3		14.8 ± 2.5		19.5 ± 4.7		10.9 ± 2.9				
NH_4^+ (μg/g)	0-20	3.21 ± 0.30		4.71 ± 2.0		3.13 ± 0.92		3.0 ± 0.70		.728	**.002 ****	NA[(a)]
	20-25	23.1 ± 15		6.86 ± 1.0		5.05 ± 1.4		10.3 ± 2.8				
Mineral N (μg/g)	0-20	61.5 ± 7.4		65.8 ± 8.1		62. ± 7.8		54.4 ± 2.0		.940	**<.001 *****	.678
	20-25	39.4 ± 17		21.6 ± 3.3		24.6 ± 4.1		21.1 ± 4.7				
Soluble organic C (μg/g)	0-20	115 ± 19	b	164.4 ± 20	a	125 ± 14	b	125 ± 6.4	b	**.004 ****	**<.001 *****	**.044 ***
	20-25	58.4 ± 9.4	b	46.0 ± 14	b	46.3 ± 19	b	68.4 ± 5.2	a			
Soil biochemical properties												
Δ NO_3^-(μg/g)	0-20	15.7 ± 4.2		7.79 ± 10		4.75 ± 17		9.58 ± 4.4		.442	.091	NA[(a)]
	20-25	27.5 ± 22		16.4 ± 26		17.5 ± 12		19.2 ± 19				
Δ NH_4^+ (μg/g)	0-20	1.02 ± 0.72		1.51 ± 1.0		0.988 ± 1.1		1.90 ± 0.71		.992	.696	.627

	20-25	-14.8 ± 16		0.089 ± 1.8		1.40 ± 1.4		-2.47 ± 3.6				
Δ Soluble organic C (µg/g)	0-20	-24.9 ± 24	B	-62.8 ± 25	A	-7.67 ± 18	B	-2.13 ± 9.6	B	**.012***	**<.001 *****	.149
	20-25	14.2 ± 16		23.9 ± 18		33.2 ± 13		20.1 ± 8.8				
Microbial biomass N (µg/g)	0-20	14.6 ± 1.9	AB	8.77 ± 4.2	B	16.3 ± 1.7	AB	17.9 ± 1.6	A	**.010***	.119	**.077.**
	20-25	3.34 ± 2.1		3.61 ± 2.7		5.27 ± 1.5		3.13 ± 0.66				
Δ Microbial biomass N (µg/g)	0-20	2.64 ± 4.3	B	13.0 ± 3.4	A	3.30 ± 1.3	B	5.35 ± 2.4	B	**.031***	**.021***	.466
	20-25	2.41 ± 1.1		3.96 ± 3.5		-0.861 ± 3.0		1.65 ± 0.86				
Microbial biomass C (µg/g)	0-20	241 ± 23	B	176 ± 60	B	272 ± 19	AB	296 ± 21	A	**.012***	.929	**.072.**
	20-25	186 ± 16		196 ± 27		203 ± 25		178 ± 4.3				
Δ Microbial biomass C (µg/g)	0-20	-100 ± 33	AB	-36.5 ± 51	A	-134 ± 13	B	-146 ± 17	B	**.047***	**.065.**	.369
	20-25	-12 ± 10		-115 ± 30		-150 ± 42		-119 ± 18				
µg C-CO_2/g.day	0-20	7.94 ± 0.20		8.06 ± 0.70		9.15 ± 0.20		8.74 ± 0.44		**.063.**	**.004 ****	**.061.**
	20-25	6.34 ± 0.23		6.57 ± 0.29		5.98 ± 0.31		6.10 ± 0.33				
***q*CO_2**	0-20	0.577 ± 0.06		3.49 ± 2.9		0.578 ± 0.03		0.509 ± 0.04		.130	.079	.046
	20-25	0.589 ± 0.05		0.603 ± 0.08		0.531 ± 0.09		0.584 ± 0.04				

Data are presented as mean ± standard error (n = 4) for each treatment and soil layer depth. Different letters indicate significant differences between treatments based on Tukey's test (*p*-value ≤ 0.05). Upper case letters indicate treatment effects across both depths, while lower case letters indicate treatment effects with each depth. Levels of statistical significance are indicated as follows: ****P* ≤ 0.001, ***P* ≤ 0.01, **P* ≤ 0.05, and ·*P* ≤ 0.1 (trend). A non-parametric Kruskal-Wallis test was used for parameters marked with (a). Abbreviations: T: treatment, D: depth, TxD: treatment by depth interaction, NA: not applicable.

The substrate-induced respiration (SIR) was 24% higher in the RCW-HD treatment than in the CTL control and 32% higher than in the CMP treatment ($P < .05$) (**Table S3** in **Supplementary data**). Carbohydrate-induced respiration was 4% higher in RCW-LD compared to CMP ($P < .05$). Respiration induced by phenolic compounds was 2% higher in CTL than in the RCW-HD treatment ($P < .05$), while surface soils showed 10% higher respiration than subsurface soils ($P < .001$). Organic acid-induced respiration was 4% higher in RCW-HD than in CMP; and surface soils had 5% higher respiration than subsurface soils. The depth effect was influenced by specific phosphorus derivative substrates that were increased in the subsurface, including ATP with an increase of 10%, apatite with 18%, Na_2HPO_4 with 17% and phytate with 19% compared to the surface.

3.2. RCW-HD favours assimilatory nitrate reduction gene abundances in the surface soil

From the KEGG annotation, 47 functional genes related to the N cycle were identified (**Table S4, Table S5**, **Supplementary data**). The *nifD* gene (N fixation) was 89% more abundant in the CTL than in the CMP ($P < .05$), while no significant difference was observed compared to the RCW treatments (**Fig.**

283 **2a**). In addition, *nifD* was 150% higher in the subsurface than in the surface layer ($P < .01$) (**Fig. 2b**).
284 Similarly, *nifK* increased by 191% in the subsurface ($P < .05$).
285 The *amoA-pmoA* gene (nitrification) was 42% higher in CTL than in RCW-HD ($P < .05$) and 228% higher
286 in the subsurface ($P < .001$). *pmoC-amoC* increased by 112.3% (CMP) and 71.8% (CTL) compared to
287 RCW-HD ($P < .05$) and by 300% in the subsurface ($P < .001$). *pmoB-amoB* and *hao* genes also showed
288 significant depth effects, increasing by 471% and 65%, respectively ($P < .001$). Conversely, *NIT-1* was
289 19% more abundant in the surface layer ($P < .001$). The *nosZ* and *nirS* (denitrification) genes showed
290 treatment effects, with *nosZ* 14% higher in CTL compared to RCW-HD ($P < .05$) and *nirS* 32% higher in
291 CMP compared to RCW-LD ($P < .05$). Both genes were more abundant in the surface layer, increasing
292 by 48% and 46%, respectively ($P < .001$). Other denitrification genes (*norB, narI, norC*) showed
293 significant depth effects, with increases of 101%, 42% and 57% in the surface layer, respectively ($P <$
294 $.01$; $P < .001$). In contrast, the *narG* gene showed an increase of 32% in subsurface layer ($P < .001$).
295 The *nrtA* and *nrtB* (assimilatory nitrate reduction) genes were significantly higher in RCW-HD
296 compared to CTL, with increases of 14% ($P < 0.01$) and 12% ($P < .001$), respectively. Both genes showed
297 higher abundance in surface soils with increases of 16% (*nrtA,* $P < .05$) and 32% (*nrtB*, $P < .05$),
298 respectively. The interaction between treatment and depth was significant ($P < .05$), with RCW-HD
299 showing the highest abundance in the surface layer. *nrtC* was 12% higher in RCW-HD compared to CTL
300 ($P < .05$) and 69% higher in the surface ($P < .001$). Similar depth effects were observed for genes such
301 as *NRT2, narB* and *nasC*, with increases of 29%, 107% and 22% in the surface layer, respectively (*NRT2,*
302 $P < .001$; *narB,* $P < .001$; *nasC*, $P < .01$). The *nirD* gene (dissimilatory nitrate reduction) was 24% higher
303 in RCW-LD compared to CMP ($P < .05$), with a 59% increase in subsurface ($P < .001$). A similar depth
304 effect was observed for *nrfA* (+19%, $P < .001$). In contrast, *napA, napB* and *nirB* were more abundant
305 in surface soils, with increases of 90%, 173% and 72%, respectively.
306 The *gdhA* gene (ammonification) showed a significant depth and treatment depth interaction effects
307 ($P < .01$), with a 15% increase in the subsurface ($P < .001$). In addition, the significant treatment-depth

interaction ($P < .01$) suggests that the distribution of *gdhA* abundance between depths differed between treatments, with subsurface values in CMP and RCW-HD significantly different from surface values, highlighting depth-specific responses to management practices. Depth effects were also observed for other genes such as *GDH2* and *gltB,* which increased by 48% (*GDH2*, $P < .01$) and 6% (*gltB,* $P < .001$) in surface soils. In contrast, *gudB* and *GLUD1-2* increased by 41% and 50% respectively in subsurface soils ($P < .001$). Other N-cycling genes, such as the *arcC* gene, showed a pronounced depth effect, with a 74% higher relative abundance in the subsurface ($P < .001$). A significant interaction between treatment and depth was also observed ($P < .05$), indicating that the relative abundance of *arcC* varied across depths depending on the treatment. In particular, the subsurface showed consistently higher abundances across all treatments, whereas the surface abundance varied more between treatments. Other genes, such as *glnA* and *CPS1* increased by 18% and 183%, respectively in subsurface soil ($P < .001$). In contrast, *cah* and *cynT* increased by 101% (*cah,* $P < .01$) and 48% (*cynT,* $P < .001$) in surface soil.

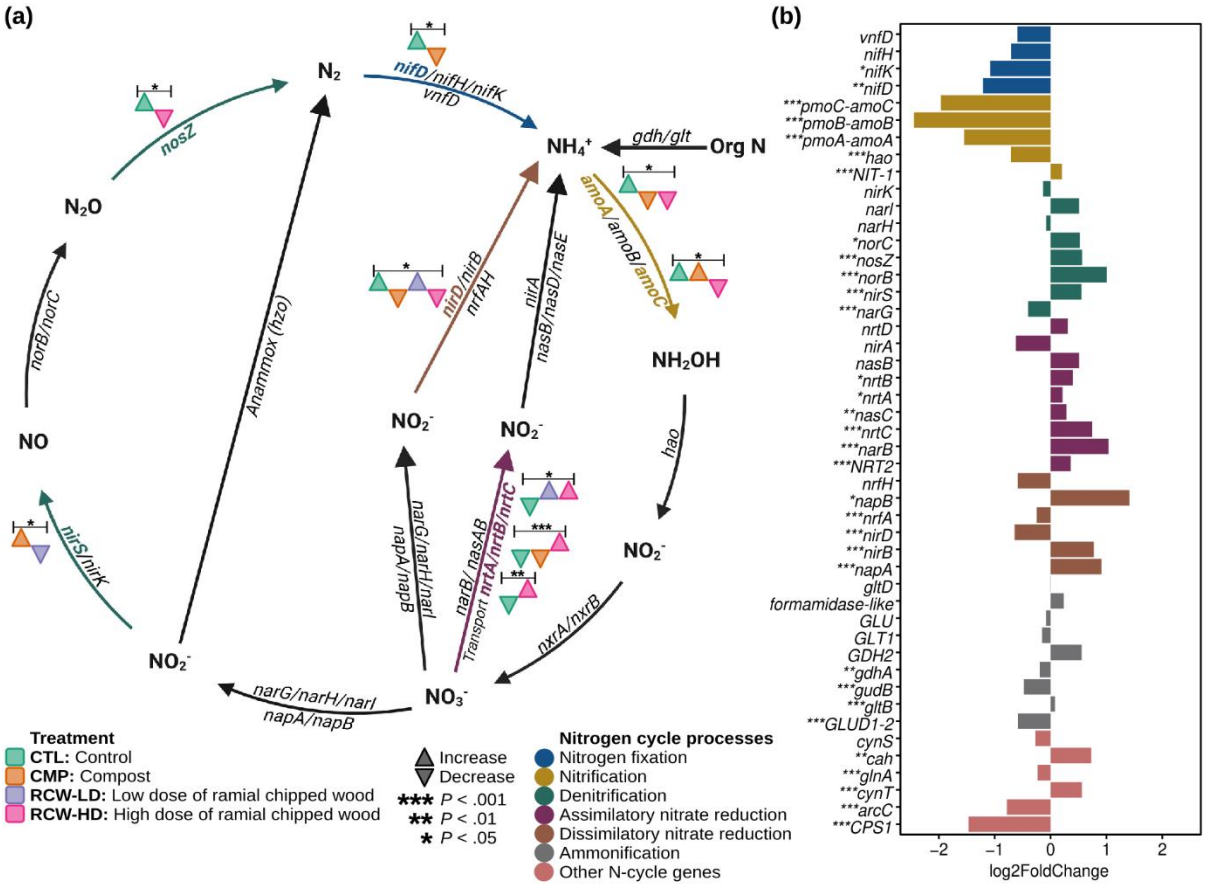

Fig. 2. Effect of organic fertiliser and soil layer on functional genes involved in soil N cycling. (a) Nitrogen cycling processes identified through metagenomic shotgun sequencing. Coloured arrows indicate N cycling processes where treatment effects were significant. ▲ and ▼ indicate significant increases and decreases in relative abundance, based on LME modelling. Genes with significant LME results are labeled in asterisks. **(b)** Bars represent log2-fold changes in functional genes involved in the N cycle across soil layer depth, with the subsurface (20-25 cm) as reference, coloured by N cycle processes. Positive bars indicate higher gene abundance in the surface layer (0-20 cm), while negative bars indicate higher abundance in the subsurface. Statistically significant values are indicated by asterisks: ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$.

3.3. Distinct microbial communities and key taxa between soil depths and management practices

Soil layer affects bacterial, archaea and fungal diversity and community composition

The most notable result for microbial alpha diversity was the significant influence of the soil layer (**Table S6, Supplementary data**). As expected, bacterial diversity and richness were higher in the surface layer, with the Shannon index showing slightly lower diversity in the CMP treatment in both

layers. Beta diversity analysis showed a strong soil layer effect (PERMANOVA, R^2 = 0.180, F = 6.18, *P* = .001, **Table S7, Supplementary data**). Principal Coordinate Analysis (PCoA) showed a clear separation by soil layer, with 43.4% of the variance explained by PCo1 (**Fig. 3a**). Subsurface components showed a more scattered distribution compared to the surface layers. Archaeal alpha diversity (Shannon index) was higher in the surface layer, while Chao1 and ACE indices indicated the highest richness in CTL (**Table S6, Supplementary data**). Beta diversity showed a strong soil layer effect (PERMANOVA, R^2 = 0.543, F = 32.373, *P* = 0.001, **Table S7, Supplementary data**), with PCoA showing a variance of 64.2% in PCo1 (**Fig. 3b**). Fungal alpha diversity (Shannon index) was lower in the surface layer, while richness showed no significant differences between treatments or layers. (Beta diversity analysis indicated a significant effect of soil layer depth on fungal community composition (PERMANOVA, R^2 = 0.251, F = 10.059, *P* = 0.001, **Table S7, Supplementary data**). PCoA highlighted this effect, with PCo1 accounting for 58.9% of the variance and a scattered distribution in the surface layer (**Fig. 3c**).

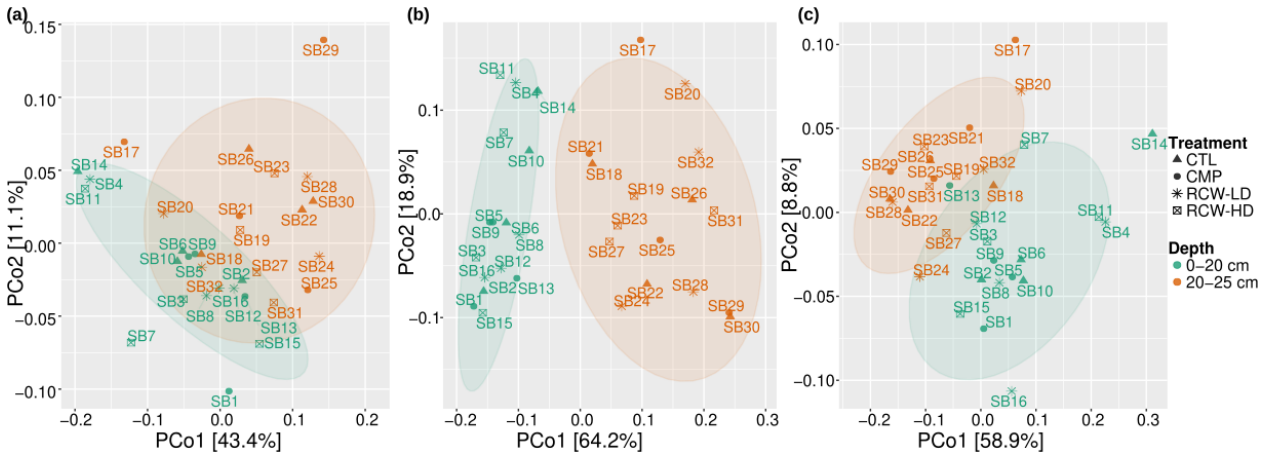

Fig. 3. Microbial community beta diversity across treatments and soil depths. Principal coordinate analysis (PCoA) based on Bray-Curtis distance matrices based on soil layer factor of **(a)** Bacteria, **(b)** Archaea, and **(c)** Fungi.

RCW application increases various taxa related to N-fixation and assimilatory nitrate reduction

To assess the impact of organic management systems and soil depth on N cycling, we analysed key bacterial (**Table S8, Supplementary Data)** and fungal taxa (**Table S9, Supplementary Data**). RCW treatments, particularly at the surface, increased the relative abundance of N-fixing genera such as

355 *Terrihabitans, Ferriphaselus, Azospira, Rhodopseudomonas, Hyphomicrobium* and *Neorhizobium,*
356 while *Ensifer* was favoured in the CTL treatment. The nitrifying genera *Methylomonas* and
357 *Methylogaea* responded differently, with CTL reducing their abundance at the surface. Denitrifying
358 genera showed contrasting responses, *Ensifer* and *Aminobacter* were more abundant in CTL, whereas
359 RCW treatments promoted *Hyphomicrobium, Agrobacterium, Sulfurimicrobium, Neorhizobium,*
360 *Azospira, Panninobacter* and *Tiobacillus.* For assimilatory nitrate reduction, RCW-HD increased
361 *Terrihabitans, Agrobacterium* and *Labrys* abundances, while *Neorhizobium, Nisaea* and *Methylosinus*
362 were increased in RCW-LD. However, CTL favoured *Paenarthrobacter, Aminobacter, Arthrobacter,*
363 *Pseudarthrobacter* and *Ensifer*. For dissimilatory nitrate reduction, *Arthrobacter* and
364 *Pseudarthrobacter* were more abundant in CTL, while *Methylomonas* was less abundant. In
365 ammonification, *Hyphomicrobium, Pseudorhodoplanes, Aminobacter* and *Agrobacterium* were the
366 main genera affected by treatments, with RCW treatment generally promoting their abundance,
367 except for *Aminobacter,* which was more abundant in CTL. No significant treatment effects were
368 observed in archaeal taxa, though *Haloarcula* and *Halosimplex* were more abundant at the surface.
369 Among the fungi, *Aspergillus* was more abundant in RCW-HD, particularly at the surface, while
370 *Thermothelomyces* increased under RCW-LD. In contrast, *Thermothielavioides, Ustilago* and *Botrytis*
371 increased on the surface in CMP. *Fusarium, Aspergillus, Thermothelomyces* and *Neurospora* showed a
372 surface preference, whereas *Pyricularia, Purpureocillium, Akanthomyces, Zymoseptoria* and *Fulvia*
373 were more abundant in the subsurface.
374
375 *3.4. Influence of functional and taxonomic composition on soil chemical and biochemical properties*
376 In the RDA model (**Fig. 4a**), SOC, ATP-induced respiration, SIR, and microbial biomass changes were
377 the driving factors to explain treatment-influenced N cycling genes. These values accounted for
378 66.81% of the explained variation (R^2 = 66.3%, *P* = .001). SOC and APT-induced respiration showed
379 both positive and negative relationships with the N cycling genes. Specifically, SOC was positively

associated with the denitrification gene *nosZ* and the assimilatory nitrate reduction genes (*nrtBC*) and negatively associated with the N-fixation gene (*nifD*), and the dissimilatory nitrate reduction gene (*nirD*). Conversely, ATP-induced respiration was negatively associated with *nirD, nifD* and *pmoA-amoA*. Furthermore, the denitrification gene *nirS* showed a positive correlation with changes in N and C microbial biomass. SIR showed a strong positive association with the assimilatory nitrate reduction genes (*nrtAB*) and a negative association with the nitrification gene *pmoC-amoC*. The results of the PERMANOVA analysis further confirmed the significant role of environmental variables in shaping the gene community. Specifically, SOC was found to explain a significant proportion of the variation in gene dissimilarities (R^2 = 0.583, F = 47.32, *P* = 0.001). Furthermore, SIR also significantly influenced gene composition (R^2 = 0.045, F = 3.66, *P* = 0.035).

The second RDA model (**Fig. 4b**), *nirD, pmoC-amoC, nosZ* and *nifD* were the driving factors in explaining the treatment-affected soil properties, accounting for 34.85% of the variance (R^2 = 32.2%, *P* = .001). *nirD* showed a positive association with changes in soluble organic, while a negative relationship was observed with phenol-induced respiration and changes in microbial biomass N. The *pmoC-amoC* gene showed a negative association with microbial biomass C and SIR. The *nosZ* gene showed a positive relationship with soluble organic C and phenolic compound induced respiration and a negative relationship with changes in soluble organic C and ATP-induced respiration. *nifD* also showed a negative association with changes in microbial biomass N and a positive association with ATP-induced respiration. The results of the PERMANOVA analysis further supported these findings, showing that the N cycling genes themselves significantly explained the variation in soil properties. Specifically, *nifD* (R^2 = 0.320, F = 73.77, *P* = 0.01)*, pmoC-amoC* (R^2 = 0.483, F = 111.29, *P* = 0.01), and *nosZ* (R^2 = 0.059, F = 13.80, *P* = 0.01) had strong, significant effects on the dissimilarities in the dataset, while *nirD* also showed a significant influence (R^2 = 0.020, F = 4.55, *P* = 0.025).

Pearson correlations (**Fig. 4c**) show that nitrification genes showed a positive correlation (r>0.5, *P*<0.05) with phytate-induced respiration, but a negative correlation (r<-0.5, *P*<0.05) with SOC,

405 microbial biomass N, NO_3^-, respiration, soluble organic C, total N, and phenolic compound-induced
406 respiration. In contrast, certain denitrification genes and genes involved in assimilatory and
407 dissimilatory nitrate reduction showed a positive correlation ($r>0.5$, $P<0.05$) with SOC, microbial
408 biomass N, NO_3^-, respiration, total N, and phenolic compound-induced respiration. In addition,
409 ammonification genes were positively correlated ($r>0.5$, $P<0.05$) with SOC and microbial biomass N.
410 The top 45 bacterial genera and 12 fungal genera that were most significant in the farming practices
411 factor and those that had an interaction with depth, as obtained by the GLMM model, were selected
412 for further analysis **(Fig. 4d)**. We observed that genera involved in assimilatory and dissimilatory
413 nitrate reduction, as well as some N-fixing and denitrifying genera, showed positive correlations
414 ($r>0.5$, $P<0.05$) with SOC, microbial biomass N, respiration and phenolic compound induced
415 respiration. In contrast, negative correlations ($r<-0.5$, $P<0.05$) were observed between some taxa
416 involved in assimilatory and dissimilatory nitrate reduction, denitrification and N fixation, as well as
417 respiration induced by phytate, ATP, and apatite.

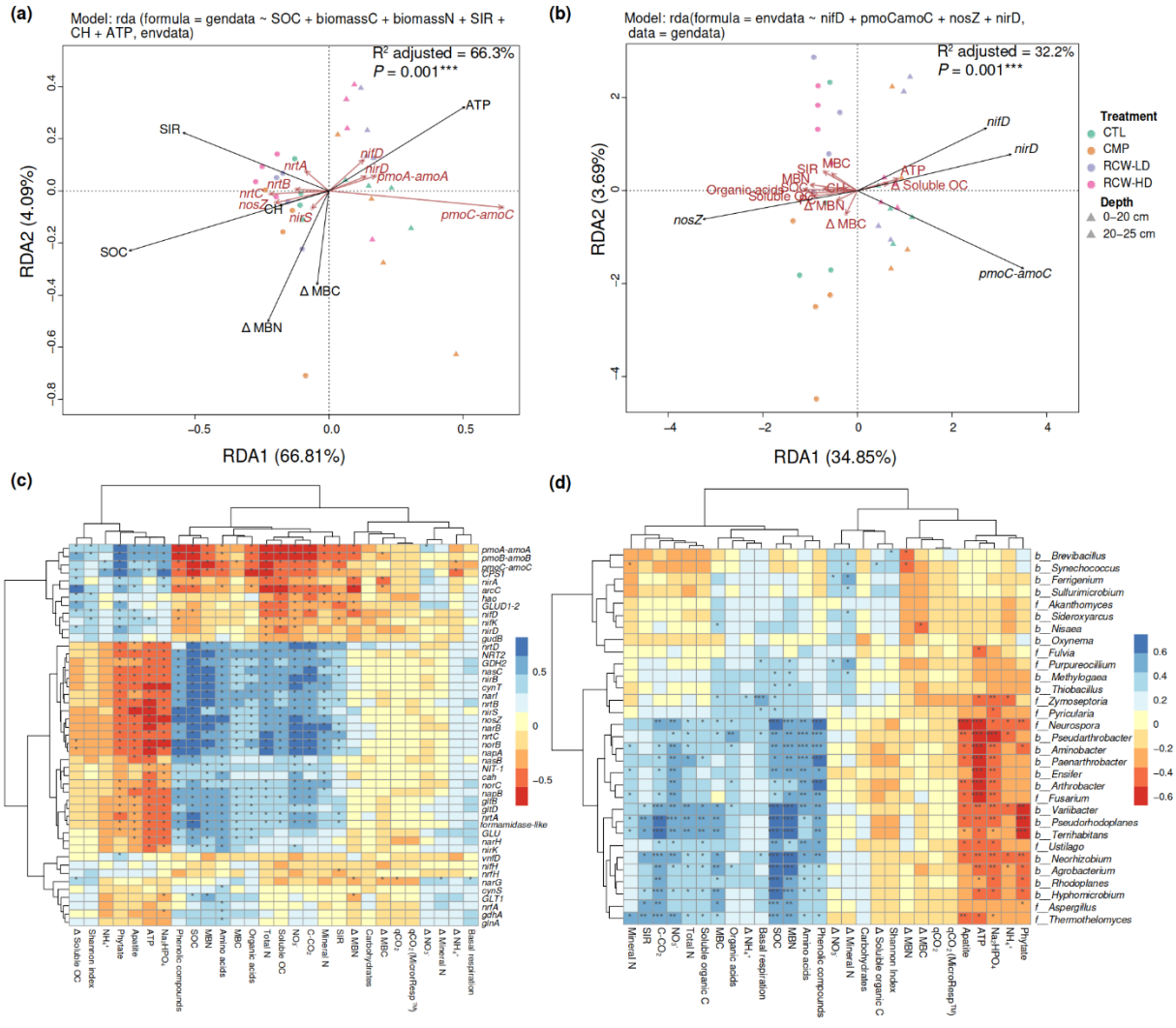

Fig. 4. Relationship between N cycle genes, microbial communities, and soil chemical and biochemical properties. (a) RDA triplot of the model where genes were treated as dependent variables. In the RDA analysis, the black arrows represent the soil chemical and biochemical properties, and red arrows represent genes. The length and direction of the arrows indicate the strength and direction of the relationship between the genes and the environmental gradients represented by the RDA axes. Sample plots are shown in circles and triangles colored by treatment; **(b)** RDA triplot of the model where genes treated as independent variables. Black arrows represent genes, and red arrows represent soil chemical and biochemical properties **(c)** Pearson correlation heatmap showing the relationships between genes and soil chemical and biochemical properties; **(d)** Pearson correlation heatmap showing the relationships of bacterial and fungal genera (most significant by treatment) with soil chemical and biochemical properties. Positive correlations are shown in blue and negative in red. Abbreviations: SOC (soil organic carbon), SIR (substrate-induced respiration), MBC (microbial biomass carbon), ΔMBC (microbial biomass carbon changes), MBN (microbial biomass nitrogen), ΔMBN (microbial biomass nitrogen changes), soluble OC (soluble organic

carbon), Δsoluble OC (soluble organic carbon changes) CH (carbohydrate-induced respiration), PC (phenolic compound-induced respiration), C-CO_2 (respiration).

4. Discussion

This study highlights the potential of RCW, a C-rich organic amendment, to shape soil microbial communities and N cycling in conservation agriculture systems. The data collected during the second cultivation year after RCW incorporation captured a critical phase of short-term microbial and biogeochemical responses, during which microbial activity and organic matter turnover are typically highest. As such, our findings provide valuable early insights and a quantitative baseline to inform the refinement of hypotheses and targeted analyses as this long-term experiment progresses. During this initial phase, we found that RCW had a distinct influence on microbial community structure and functional gene abundance compared to traditional compost. These results provide new insights into how the type and dose of organic amendments can drive key soil processes relevant to sustainable fertilisation strategies.

Consistent with our hypothesis, the application of large amounts of RCW in a no-till system resulted in an increase in SOC concentration, which was associated with an increase in microbial biomass C, while maintaining tomato yields levels. Although initial yield losses due to N immobilisation have been reported (Soumare et al., 2002), improvements were observed in the second cropping cycle, probably due to nutrients release. Similar positive effects on tomato yield following RCW application were also reported by (Robert et al., 2014). To our knowledge, there are no previous studies on the application of woody plant residues in a no-till organic fertilizer system on soil N processes. However, some studies have shown that the integration of C-rich plant material into topsoils promotes soil structure and soil fungal activity, leading to increased stability of SOC levels (Kok et al., 2022; Daassi et al., 2024), long-term soil fertility and nutrient retention (Clocchiatti et al., 2021; Li et al., 2024), N uptake (Fontana et al., 2023), and protection against soil erosion (Robichaud et al., 2013). In addition, no-till

455 practices have been shown to increase SOC storage while reducing oxidation and improving soil
456 aggregation and water infiltration (Gao et al., 2016; Bösch et al., 2022). While compost-treated soils
457 had higher concentrations of soluble organic C, microbial biomass C and N were lower compared to
458 other treatments, in contrast to findings from previous studies (Goyer et al., 2022). These results
459 highlight the complexity of microbial responses to organic amendments and the importance of
460 considering both substrate availability and microbial assimilation efficiency when assessing soil
461 fertility.
462 In our study, bacterial diversity decreased in surface soils treated with RCW-LD, while a decrease was
463 observed in subsurface soils treated with compost. These results contrast with previous studies that
464 reported no significant changes in bacterial diversity following compost application (Shu 2023).
465 However, our plant-based compost without manure differs from those with higher wood content or
466 municipal organic waste, which tend to increase bacterial diversity (Goyer et al., 2022). It is important
467 to note that our compost is plant-based and does not contain manure, making it different from those
468 in previous studies. In surface soils, archaeal diversity decreased significantly with compost
469 amendment, which coincided with an increase in soluble organic C. This suggests that the increased
470 availability of labile C altered competitive dynamics, favouring the diversity of bacteria over that of
471 archaea, which occupy more specialised ecological niches. Fungal communities, however, remained
472 unaffected by the agronomic practices tested, suggesting resilience or stability in their composition
473 regardless of the management approaches applied. These findings are consistent with other studies
474 suggesting that soil bacterial diversity is more sensitive than fungi to changes in the soil environment
475 caused by agricultural practices (Y. Li et al., 2020; Bebber and Richards, 2022; Shu et al., 2022).
476
477 *4.1. Biological N fixation*
478 Our results showed a higher *nifD* gene abundance in the CTL treatment, suggesting that the N-rich
479 organic fertiliser may create favourable conditions for N-fixing microbes. The lower *nifD* abundance in

480 the compost coincides with higher organic C availability and with high microbial N content, indicating
481 differences in microbial competition and enzymatic activity (Hoang et al., 2022; Zeiner et al., 2024) in
482 compost soils. This may have increased microbial access to organic N pools and may have reduced the
483 need for incorporation of highly stable N_2 pools. RCW-HD, with the highest SOC, did not show higher
484 *nifD* abundance. N fixation is a P-intensive process due to its high energy demand (Reed et al., 2007),
485 and RCW may not have provided sufficient available P to support increased diazotrophic activity.
486 Positive correlations of *nifK* and *nifD* with apatite, phytate and ATP-induced respiration reinforce the
487 critical role of P availability in supporting N fixation. We also found higher *nifD* and *nifK* abundances
488 in subsurface soils with lower C availability, similar to the findings of Wang et al., (2016) who found
489 higher abundances of N fixation communities in deeper soil layers in a humid subtropical monsoon
490 region. In contrast, Reardon et al. (2014) reported a lower *nifH g*ene abundance in subsurface layers
491 of Mediterranean fallow soils, highlighting the role of ecological dynamics. Interestingly, high free-
492 living N fixation has been described in karst soils low in N and organic C, where diazotrophs adapt to
493 nutrient-poor environments with reduced competition for resources (Tang et al., 2021), a pattern
494 observed in our results.
495 The increased abundance of several N-fixing genera in the no-till RCW treatment suggests that
496 diazotrophs are sensitive to standard agricultural practices, including fertilisation, as shown in a
497 previous study by J. Wang et al. (2016). We also found a positive correlation between these genera
498 and SOC, which is consistent with previous studies showing that SOC and C/N ratios influence
499 diazotroph abundance and activity (Levy-Booth et al., 2014; Mirza et al., 2014; Zheng et al., 2023), as
500 SOC provides energy for heterotrophic N fixation and helps maintain high respiration rates to protect
501 nitrogenase from oxidation (Smercina et al., 2019).
502
503 *4.2. Nitrification and denitrification*

504 The relative abundance of nitrifying genes and genera increased under compost treatment, with genes
505 showing a positive correlation with phytate-induced respiration. This suggests that the abundance of
506 highly stable organic P enhances nitrifying activity, so it appears that nitrifying bacteria can access
507 highly stable organic P pools. Other authors have found several bacterial genomes that link
508 nitrification to P solubilisation (Wu et al., 2021). In contrast, nitrification-related genes decreased
509 under high-dose RCW treatment, which had a higher SOC concentration. This suggests a controlled
510 release of N in RCW-HD treatment, which could reduce NO_3^- leaching and related emissions (Philippot
511 et al., 2007; Norton and Stark, 2011). In addition, we observed a depth-related effect, with nitrifying
512 genes being more abundant in subsurface soils. As nitrification occurs under aerobic conditions our
513 results may indicate good aeration in our subsurface soils. In contrast, a study conducted in paddy
514 soils under wetter conditions found a decrease in gene abundance at depths between 0 and 40 cm,
515 with further decrease observed at depths between 40 and 100 cm (H. Wang et al., 2017).
516 The lower abundance of denitrification genes in the no-till RCW system compared to CTL (*nosZ)* and
517 CMP (*nirS)* is consistent with high SOC in the RCW system, which may reduce denitrification processes.
518 This may be an indication of good aeration in no-till RCW, despite the increased SOC in no-till systems.
519 This is in contrast with other studies that have shown that reduced tillage typically increases
520 denitrification activity (Bösch et al., 2022), indicating an additional benefit of the RCW treatment. In
521 contrast, compost treatment, with its higher soluble organic C, provides more energy for these
522 microbes, leading to a higher abundance of *nirS.* This is consistent with previous studies showing that
523 the effect of organic amendments on denitrification depends on the type of amendment (Pereg et al.,
524 2018; Shi et al., 2019; Yin et al., 2020).
525 The results highlight the need to consider the interactions between soil C, microbial communities and
526 tillage when managing denitrification and reducing N_2O emissions in agroecosystems. Positive
527 correlations between denitrifying genera and SOC, microbial biomass N, NO_3^- and total N suggest that
528 increased C and N availability favours denitrification activity. Furthermore, correlations with

529 respiration, including that induced by phenolic compounds, suggest that respiratory activity is linked
530 to recalcitrant C release, which denitrifying microbes use for metabolic processes. As most of the
531 genes regulating this process (including *nosZ* and *nirS*) occur in SOC-rich surface soils, it appears that
532 the anaerobic conditions required for denitrification are mainly regulated by C and N availability rather
533 than position in the soil profile. However, no-till RCW soils show reduced relative abundances of *nosZ*
534 and *nirS* together with high SOC levels.
535
536 *4.3. Assimilatory and dissimilatory nitrate reduction*
537 The increase in *nrtABC* genes (assimilatory nitrate reduction) in the no-till RCW-HD system is
538 consistent with the observed high SOM and microbial biomass, suggesting a microbial response to
539 increased organic matter availability. Organic management systems are known to increase microbial
540 diversity and N retention in surface soils, particularly through enhanced assimilatory pathways (Y.
541 Wang et al., 2017). The higher SOM in no-till systems is likely to provide more accessible C and energy
542 sources for microbial processes, potentially influencing nitrate assimilation efficiency (Kuypers et al.,
543 2018; Piazza et al., 2020). Correlation analysis supports this by showing positive associations between
544 genes and bacterial genera abundance and SOC, microbial biomass N, NO_3^-, total N and respiration.
545 These results highlight the complex interactions between organic matter inputs, microbial activity and
546 nitrogen transformations in no-till systems.
547 The abundance of *nirD* (dissimilatory nitrate reduction) was 30% higher in RCW-LD compared to RCW-
548 HD, although both treatments had high SOC content. This suggests that *nirD* abundance is influenced
549 not only by total SOC content but also by its quality and availability. At a lower RCW dose, the C present
550 may be less recalcitrant and more readily accessible to dissimilatory nitrate-reducing microorganisms,
551 promoting greater *nirD* abundance. In contrast, the higher SOC content in RCW-HD may be associated
552 with a greater proportion of recalcitrant C, limiting its availability as an energy source for these
553 microorganisms. Previous studies have established that C acts as an electron donor through

554 fermentation or respiration, facilitating the reduction of NO_3^- to NH_4^+ and providing energy to the
555 microbial community involved in this process (Yoon et al., 2015; van den Berg et al., 2016).
556 Consequently, total soil C is considered a key regulator of this process, with increased C availability
557 generally enhancing this process (Cheng et al., 2022). Several studies have shown that C-rich soils
558 promote dissimilatory nitrate reduction (Morley and Baggs, 2010; X. Li et al., 2020). Our results suggest
559 that specific C fractions, rather than total SOC, may play a more direct role in modulating the microbial
560 dynamics of this pathway. Furthermore, genes involved in this N cycling process were more abundant
561 in the subsurface, probably due to low oxygen levels in the subsurface soil, consistent with previous
562 findings (Tu et al., 2017).
563 Although RCW has a high C/N ratio, our results suggest that it does not lead to strong N inmobilisation
564 after one year in the short term. Instead, we observed an increase in assimilatory nitrate reduction
565 genes and a differential abundance of dissimilatory genes depending on the RCW dose. This suggests
566 that not only the amount, but also the quality and availability of C play a role in the regulation of
567 nitrate reduction pathways. In particular, the lower abundance of dissimilatory genes at high RCW
568 dose may reflect the presence of more recalcitrant carbon, limiting its accessibility to microbial nitrate
569 respiration. These findings help to clarify how different carbon inputs modulate N dynamics in no-till
570 systems.
571
572 *4.4. Ammonification*
573 The ammonification process was also significantly influenced by soil depth in this study. The genes
574 *GDH-2* and *gltB* were more abundant in surface soils, while *gudB, GLUD1-2* and *gdhA* were more
575 abundant in the subsurface. This shift in soil layers likely reflects differences in organic matter
576 availability and microbial community composition between soil layers (Frey et al., 2022). In surface
577 soils where organic inputs are higher, ammonification is more active, resulting in higher rates of
578 organic N mineralisation into ammonium. In subsurface soils, where oxygen levels are lower,

alternative pathways such as ammonium retention via dissimilatory nitrate reduction may be favored over complete N loss via denitrification (Robertson and Groffman, 2023). These processes are essential for soil N conservation and are consistent with previous studies highlighting the role of ammonification in promoting soil fertility, particularly in organic and reduced tillage systems (Van Groenigen et al., 2015).

4.5. Microbial functional stability under organic amendments

We further explored community homeostasis by analysing patterns of correlation between microbial genes, genera and soil parameters in response to RCW and other organic amendments. Our correlation-based hierarchical clustering heatmaps revealed that induced respiration of all added P compounds, including available inorganic Na_2HPO_4, sparingly soluble apatite, and both labile (ATP) and recalcitrant (phytate) organic P forms, were positively associated with the abundance of nitrifying genes. This may reflect microbial P limitation as reported in other organic soils (Amador and Jones, 1993; Oliverio et al., 2020). In contrast, a wide range of other N-transforming genes including those involved in N fixation, assimilatory and dissimilatory nitrate reduction, and some/certain ammonification and denitrification pathways, were negatively associated with P compounds respirations. Whereas, soil parameters related to SOC, microbial biomass and activity, mineral N and response to organic substrates (e.g. amino acids, phenolic compounds and organic acids) showed positive association with this heterogeneous group of genes. This may indicate a decoupling of N and P cycling for microbial processes using SOC as a source of energy. This is sustained by observed correlations between soil parameters and microbial taxa associated with assimilatory and dissimilatory nitrate reduction, N fixation and denitrification, further supporting the emergence of functionally coherent microbial modules in response to the organic C availability associated with the different organic amendments applied.

These coherent patterns suggest the emergence of ecological guilds with predictable functional roles in response to the application of organic amendments of contrasted quality under tillage and no-tillage. The consistent associations between microbial taxa, soil parameters and functional genes suggest a degree of community-level homeostasis, where microbial functions are organized in response to changes in soil environmental conditions. This supports previous research on the resilience and functional organisation of soil microbial communities under nutrient variation (Zhang et al., 2023). Our results reinforce the evidence that organic amendments enhance microbial functionality and community-level stability, consistent with global patterns observed under organic management system (Shu et al., 2022). The interplay of microbial C, N and P cycling, influenced by soil stoichiometry, is crucial for microbial responses to organic amendments (Sinsabaugh et al., 2009; Zechmeister-Boltenstern et al., 2015; Zhang et al., 2023). Our study, highlighting the decoupling of N and P cycling, reinforces the idea that soil C:N:P ratios are central to microbial processes, especially in systems receiving organic inputs.

5. Conclusions

This study demonstrates that applying high doses of ramial chipped wood (RCW) in a no-till management system increases early-stage soil organic carbon and microbial biomass. Despite a 50% reduction in fertiliser input, the application of RCW maintained nitrogen availability and crop yield, highlighting its potential to promote nutrient efficiency in no-till systems. The increased abundance of assimilatory nitrate reduction genes indicates enhanced nitrogen retention, and the reduced abundance of nitrification and denitrification genes suggests a shift towards more conservative nitrogen cycling pathways. This is likely to be driven by improved carbon availability and aeration under RCW no-till conditions. These findings emphasise the value of RCW in regenerative agriculture by promoting internal nitrogen recirculation and reducing losses. Further research is needed to

optimise RCW application rates and to understand the long-term effects on carbon and nitrogen dynamics, particularly with regard to greenhouse gas emissions.

Author contributions: CRediT

Johana González Coria: Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review and editing, Visualization, Validation, Software. **Michelle-Danielle Ioan:** Methodology, Investigation, Formal analysis. **Pierre Hohmann:** Methodology, Validation, Writing – review and editing. **Guillem Segarra:** Investigation, Resources, Writing – review and editing. **Marina Pérez-Llorca:** Methodology, Validation, Writing – review and editing. **Maria Pérez:** Writing – review and editing, Funding acquisition. **Anna Vallverdú-Queralt:** Writing – review and editing, Funding acquisition. **Joan Romanyà:** Conceptualization, Methodology, Investigation, Validation, Writing – original draft, Writing – review and editing, Supervision, Project administration, Funding acquisition. All the authors have read and agreed to the version of the manuscript to be published.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found online at https://.... **Table S1:** Soil conditions before amendments application. **Table S2:** MicroResp™ substrate list. **Table S3:** Soil microbial activity (MicroResp™) under different management systems. **Table S4:** Description of the identified genes related to nitrogen cycle. **Table S5:** Relative abundance (%) of genes involved in the nitrogen cycle. **Table S6:** Alpha diversity of bacteria, archaea and fungi based on Chao1, ACE and Shannon indices for each treatment and soil layer depth. **Table S7:** Permutational multivariate analysis of variance (Permanova) results of beta diversity. **Table S8:** Relative abundance (%) of the top 45 most significant bacterial genera by treatment and its interaction with soil depth. **Table S9:** Relative abundance (%) of the most important fungal genera in all investigated factors.

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