



# Biological rehabilitation of iron ore mine tailings enhanced by organic substrate application

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## Abstract

**Purpose** Application of organic substrates to rehabilitate mine tailings is a cost-effective and commonly employed strategy. Although the remediation efficiency of various amendments has been investigated, the effectiveness of organic substrate in enhancing the microbiome in iron ore mine tailings remains unknown.

**Methods** This study hypothesised that organic substrate could enhance the root growth of perennial ryegrass (*Lolium perenne*) over 60 days under controlled glasshouse conditions in two distinct iron ore mine tailings (A and B). The impacts of organic substrate on physicochemical properties of tailings, root architecture, bacterial communities, and the abundance of C- and N-cycling genes were investigated.

**Results** In contrast to control, tailings treated with organic substrate at 75% (w/w), the concentrations of diethylenetriamine-pentaacetic acid (DTPA)-extractable Cr and Zn increased (38% and 54%), in tailings A, but remained the same in tailings B, whereas DTPA-extractable Cu and Fe increased (3-fold) and Mn (2-fold) decreased regardless of tailings type. Compared with the control, organic substrate application at 75% increased perennial ryegrass root biomass, length, and average root diameter in both tailings. Further, organic substrate application at 75% showed a substantial increase in both microbial biomass carbon and microbial respiration. The relative abundance of Proteobacteria and Bacteroidetes decreased, while that of Actinobacteria increased, with an increasing rate of organic substrate (75%). Amylose degradation was the least prominent process (*SusB*) in C cycling, whereas denitrification (*narG*, *nirK*, *nosZ*, *nrfA*) was the most prominent process in N cycling in both tailings.

**Conclusion** Overall, compared with tailings A, tailings B provided a better environment for plant growth and microbiome abundance and activity, with the addition of organic substrate further improving tailings B. This study highlights the potential of organic substrate use in rehabilitation of iron ore mine tailings via enhancing their biological attributes; however, further research is required to validate these findings under field conditions.

**Keywords** Organic substrate · Metals · Microbial diversity · Roots · Tailings

## 1 Introduction

Mining is vital to the world economy, accounting for 6.9% of the global gross domestic product. However, mining is associated with the accumulation of hazardous waste that can easily impact the surrounding environment (Araujo et

al. 2022). It is estimated that approximately 7 billion tonnes of mine tailings are generated each year globally (Marín et al. 2022), of which 14 million tonnes of tailings are from iron ore beneficiation. Revegetation of these tailings represents an economic and environmental challenge due to (i) accelerated rate of iron ore tailings generation over the past two decades (Aredes et al. 2025), and (ii) lack of proper physicochemical and biological characterisation that would enable the creation of a viable growth medium for plants and their associated soil microbial communities. Iron ore tailings have physical properties such as fine texture, poor aeration, high mechanical resistance and poor water-holding capacity (Sun et al. 2018) that are unfavourable for root penetration and microbial colonisation. Additionally, hematite iron ore

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tailings contain only trace amounts of organic carbon and nitrogen. Elevated concentrations of metals, including Fe and Mn, make these tailings quite hostile to plant growth.

An eco-engineering approach has been advocated to convert tailings into productive growth media, aiming to accelerate the development of physicochemical and biological properties and functions similar to those of natural soils (Chaturvedi et al. 2023). This would result in an attractive, financially affordable, and long-term biological platform (Bolan et al. 2014) to improve mine tailings making them a sustainable growth medium for revegetation by applying suitable organic matter, such as peat, organic substrate or biochar (Lima et al. 2025). This application may prime and accelerate mineral weathering and water-stable aggregate formation (Wu et al. 2019), thus alleviating physicochemical constraints (Al-Lami et al. 2019) and improving the biological properties of the tailings for plant establishment and growth by enhancing nutrient availability and microbial activity (Huang and Imran 2025).

Numerous studies have demonstrated that a wide range of organic materials can be used as effective amendments for mine tailings rehabilitation, including biosolids (Conesa et al. 2025), compost, woodchips, manure, and sewage sludge (Liu et al. 2026b), as well as biochar (Liu et al. 2026a). These organic substrates have been applied across diverse tailings types – such as gold, copper, iron ore, and coal mine tailings – and consistently supplement exogenous nutrient availability, stimulate microbial growth and enzyme activity, enhance pH buffering capacity, and support plant establishment (Hu et al. 2026).

The abundance of neutrophilic microorganisms in the amended tailings is strongly influenced by the availability of organic matter. Their abundance and diversity are sensitive to environmental stressors, serving both as indicators of stress (low initial abundance) and as signs of subsequent ecological improvement in the mine tailings for plant growth following amendment (increased abundance over time). Further, organic substrate directly influences the physicochemical properties of tailings, such as pH and metal/loid solubility by effectively immobilising metal ions through adsorption and complexation (Huang et al. 2016). Abundant humic substrates in organic substrates contain many organic functional groups, such as carboxyl, carbonyl and phenolic groups, which can bind metal ions through complexation (Tsang et al. 2013). In addition, some fungal taxa, such as *Ascomycota* associated with organic substrate, can immobilise heavy metals through a biosorption mechanism (Huang et al. 2016).

It has been suggested that fast-growing grasses, such as perennial ryegrass (*Lolium perenne* L.), are effective in initial revegetation of mine tailings because they have (i) fibrous root systems (Brajević et al. 2026; Wojtowicz et al.

2026) resulting in a large root surface area per unit volume of growth substrate, and (ii) the capacity to grow in a range of adverse climatic conditions. However, the suitability of grass species is highly site-specific and depends on tailings mineralogy, geochemistry and local climate. Many studies have tested the growth of perennial ryegrass in different types of tailings, including bauxite residue (Quinteros et al. 2023) and copper tailings (Alvarenga et al. 2008; Wang et al. 2024), gold tailings (Ai et al. 2020) and poly-metallic tailings in semiarid climates (Gu et al. 2017). These examples highlight that grass performance varies with tailings characteristics and environmental conditions, reinforcing the need for site-specific evaluation when selecting species for rehabilitation.

Most research has focused on non-ferrous tailings, such as copper, gold, and bauxite residues, and their rehabilitation via a range of eco-engineering approaches, including organic amendments (such as composts and biochar) and microbial inoculant application. There is a significant research gap in the rehabilitation of iron ore tailings, particularly hematite-dominant tailings from Western Australia, for which only limited studies exist despite their unique physicochemical constraints (low organic carbon, high Fe/Mn, poor structure, and low nutrient availability). Only a limited number of studies have examined the extent of influence of organic amendments on microbial activity, nutrient cycling, and root development in iron tailings, and almost none have evaluated how organic substrates affect both the microbiome and functional gene abundance. Therefore, this gap limits our understanding of how to biologically rehabilitate iron ore tailings and whether organic substrate can accelerate the development of soil-like conditions in the short term. Therefore, this study aimed to determine how increasing rates of organic substrate influence (i) physicochemical properties, (ii) microbial biomass, bacterial community composition, functional C- and N-cycling genes, and (iii) root traits of perennial ryegrass in selected two contrasting iron ore tailings.

## 2 Materials and methods

### 2.1 Materials

The tailings from two iron ore mines (A and B) were collected from Pilbara (Western Australia) (20° 47' 51" S, 119° 25' 46" E) (see Supplementary Material Fig. S6), which were considered as hematite-dominant, and low-grade iron ore beneficiated by a wet processing method. Commercially produced garden waste-based organic substrate (CM) was used as the amendment to the tailings, and the characteristics are stated in Table S1a and S1b.

## 2.2 Experimental design

A glasshouse pot experiment was set up following a randomised complete block design (RCBD) with factorial arrangement of treatments and three replicates. Pots were placed in a temperature-controlled glasshouse at the University of Western Australia (31° 59' 9.6" S, 115° 49' 19.2" E). The average temperature was 26/15°C (day/night) during the growth period, with a relative humidity of 74%. The pots (2 L) were filled with treatment substrates [mine tailings, organic substrate/tailings mixtures, organic substrate (0%, 50%, 75% and 100% w/w, respectively)]. All treatments received 0.5 g kg<sup>-1</sup> of mineral fertiliser (N 10%, P 7%, K 4.5%, Fe 2.7% and Mn 0.43%, all in w/w) (Troforte Innovations Pty. Ltd, Perth, Western Australia). The pots were watered to 60% of water holding capacity and left to equilibrate in a glasshouse for 1 week.

## 2.3 Physicochemical analysis

The pH and electrical conductivity (EC) were measured in unamended/amended mine tailings and in the organic substrate in water at a 1:5 (w/v) solution ratio (25 °C) using a standard glass electrode (ORION 5-star series meter, Thermo Electron Corporation, Massachusetts, USA). Total organic carbon (TOC) and cation exchange capacity were measured by the dichromate wet oxidation method and the 0.01 M Ag-thiourea method, respectively, following the methods of Rayment and Lyons (2010).

The plant-available fractions of Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn were assessed by extracting 10 g of each substrate with 20 mL of diethylene-triamine pentaacetic acid (DTPA) extractant (0.005 M DTPA + 0.01 M CaCl<sub>2</sub> + 0.1 M triethanol amine, adjusted to pH 7.3) (Katyal and Sharma 1991) and measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Optima 5300DV, Perkin Elmer, Shelton, Connecticut, USA).

## 2.4 Plant growth and root analysis

Five seeds of surface-sterilised healthy perennial ryegrass (*Lolium perenne* L.) were sown in prepared pots, with no thinning performed, and harvested after 45 and 60 days. The data trends for the two harvests were similar; hence, only the data for the harvest after 60 days of growth are included here. At harvest, shoots and roots were separated by cutting just above the substrate surface. Roots were washed out of the substrate column under running tap water and rinsed in deionised water. Approximately 0.2–0.5 g of fresh root sub-samples were stored in 50% v/v ethanol until scanning on a root scanner (Epson Perfection V 800 Photo, Digital ICE Technologies, Nagano-ken, Japan). Total root length and average root diameter were determined using the commercial

software package WinRHIZO 2008a (Regent Instruments Canada Inc., Quebec, Canada). Total root dry weight was determined by drying root samples at 70 °C for 72 h.

## 2.5 Preparation of plant roots for arbuscular mycorrhizal (AM) colonisation assessment

To assess AM colonisation, sub-samples (~0.25 g) of fresh roots were cut to approximately 1 cm in length and cleared by immersing in 10% (w/v) KOH for 5 days. The roots were then rinsed with deionised water, acidified, and stained with 0.05% w/v trypan blue in lactoglycerol (Solaiman et al. 2020). Then mycorrhizal colonisation was assessed microscopically using the gridline-intersect method.

## 2.6 Analysis of microbial biomass carbon (MBC)

The MBC was estimated in rhizosphere soil after 60 days of perennial ryegrass growth using the modified fumigation-extraction method. Chloroform fumigation was performed with ethanol-free CHCl<sub>3</sub> in a desiccator lined with moist paper in a dark incubation room at 25 °C for 24 h. Soluble organic carbon was extracted by shaking 10 g of soil with 40 mL of 0.5 M K<sub>2</sub>SO<sub>4</sub> on a tumbler for 1 h. The dissolved organic carbon concentration in extracts was measured using an automated total organic carbon (TOC) analyser (Shimadzu, TOC-500, Tokyo, Japan). The difference in soluble carbon between non-fumigated and fumigated samples was calculated using K<sub>EC</sub> = 0.45, a constant value for unknown soil samples at 25 °C.

## 2.7 CO<sub>2</sub> respiration measurement

CO<sub>2</sub> evolution was determined from substrate mixtures under basal conditions in a closed-chamber system. The substrate mixtures were pre-incubated at 25 °C for 7 days. Water content was maintained at 45% of water holding capacity during incubation by adding deionised water to the weight. Substrate CO<sub>2</sub> respiration was measured every second day for 14 days, then once a week from day 15 to the end of the incubation period (105 days). On each measurement day, 1 mL from the jar headspace was extracted using a syringe and inserted into an infrared CO<sub>2</sub> gas analyser (Q-S151, CO<sub>2</sub> Analyser, Qubit systems, Ontario, Canada). After sampling, the jars were opened for 30 s and then air-proofed until the next measurement. There were triplicate experimental jars for each treatment.

## 2.8 Sampling, DNA extraction and sequencing

DNA was extracted from 0.4 g of unamended/amended tailings and organic substrate samples using a Power Soil DNA

Isolation Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol, with modifications to the bead-beating and elution steps. The extracted DNA was quantified in a Qubit 3.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) to maintain at least 50 ng/μL per sample and stored at -20 °C before amplification. The triplicate samples for each treatment were amplified with the universal primer pair 341 F (5'-CCTAC-GGGNGGCWGCAG-3') (Marangi and Boughattas 2025) and 806R (5'-GGACTACVSGGGTATCTAAT-3') (Wang et al. 2022a) amended with Golay barcodes to sequence the hypervariable V3-V4 region of the 16 S rRNA gene at the Australian Genome Research Facility (Melbourne, Victoria, 3000, Australia) by using an Illumina MiSeq platform with 2 × 300 bp paired-end V3.

## 2.9 Bioinformatics

All bioinformatics analyses were carried out at the Australian Genome Research Facility. The raw gene amplicons were processed using DADA2 (version 1.20.0) with the recommended quality filtering settings (Callahan et al. 2016) such as duplicate sequences, chimeras and The reads identified as mitochondria and singletons as well as ASVs (Amplicon Sequence Variants) found in less than 1% of the reads, were eliminated Taxonomic sequence variants were clustered and classified using SILVA version 132 (Klimenko et al. 2020). There was no filtering of archaea.

Prediction of putative genes that encode C- and N-cycling enzymes was made by the in-silico Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST2) tool, as described in Douglas et al. (2020). This method infers 16S rRNA-based bacterial community data to profile metagenomic samples. PICRUST2 (Yang et al. 2023), accompanied by EPA-ng (Barbera et al. 2019), Gappa (Czech et al. 2020), Castor (Louca and Doebeli 2018) and Minipath (Ye and Doak 2009) was used to assign filtered sequencing reads to functional orthologs (KEGG orthologs; KOs) and infer functional pathways using the MetaCyc database (Caspi et al. 2016).

## 2.10 Statistical analysis

Generated data were analysed using the Genstat software package (Genstat, 19th Edition, VSN International Ltd, Rothamsted, Harpenden, UK). The two-way analysis of variance tested significant differences among different tailings and organic substrate mixtures. The Tukey HSD test was employed to compare the treatment means. The differences between the values were considered significant at  $P \leq 0.05$ . The data that did not meet the assumptions of normality and homoscedasticity were log-transformed, but

the results are presented on the original scale. In addition, data were subjected to principal component analysis (PCA) to estimate the multivariate effects of established treatments and to examine the relationships among variables. The canonical correlation analysis (CCA) was performed to explore correlations among phyla, physicochemical attributes, and microbial activity (Moneda et al. 2022).

## 3 Results

### 3.1 Physicochemical and plant-available elements in amended and unamended tailings

A significant interaction between tailings source and organic substrate rate ( $P < 0.0001$ ) was observed in pH, and organic substrate (6.38) was intermediate between the near-neutral unamended tailings pH values of A (6.55) and B (6.07). In amended tailings A, pH showed an inverse relationship with increasing organic substrate application rates 50 (CM<sub>50</sub>) and 75 (CM<sub>75</sub>) % w/w, decreasing to values of 6.51 and 6.47, respectively. In contrast, pH in tailings B increased proportionally at the same application rates, reaching 6.19 and 6.34, respectively. The values of EC showed a similar trend as pH with a significant interaction (tailings source and organic substrate rate) ( $P < 0.0001$ ). The EC value of unamended tailings A (2.54 dS m<sup>-1</sup>) was 13-fold higher than that of tailings B (0.20 dS m<sup>-1</sup>). Although CM<sub>50</sub> and CM<sub>75</sub> organic substrate treatments did not show a significant difference within each tailings source, significant differences were observed between the tailings sources at the same application rate. The cation exchange capacity increased proportionately with organic substrate application rate, and the highest (5- and 2-fold) was observed in 100% organic substrate than in unamended tailings A and B, respectively. A significant ( $P \leq 0.05$ ) interaction effect (tailings source and organic substrate rate) was observed in total organic carbon content. Unamended tailings A and B reported the lowest values, including 0.8 g kg<sup>-1</sup> and 1.0 g kg<sup>-1</sup>, and were approximately 100-fold lower than organic substrate.

The DTPA-extractable Cr concentrations in organic substrate -amended tailings A decreased (38% and 54%, respectively) with an increase (50% and 75%) in the organic substrate rate, whereas no significant differences were recorded in tailings B, making the interaction between tailings source and organic substrate rate significant ( $P \leq 0.05$ ) (Results shown in Supplementary material, Table S2). An increasing trend was observed for DTPA-extractable Zn, by 2-fold with an increase in organic substrate rate of 50% and 75% w/w only in tailings A. DTPA-extractable concentrations of Cu, Fe and Mn were not significantly influenced by the interaction between tailings source and organic

substrate rate (Results shown in Supplementary material, Table S3), but the main effect of organic substrate rate was significant ( $P \leq 0.05$ ). In general, there was an increase in DTPA-extractable Cu, Fe and Mn (2-fold) with 50% w/w of organic substrate rate and a 3-fold increase in Cu and Fe with 75% organic substrate application rate. By contrast, the tailings source was identified as the only influencing factor for DTPA-extractable Ni and Pb (Results shown in Supplementary material, Table S4), with tailings A exhibiting a 2-fold higher Ni concentration and tailings B exhibiting a 1.5-fold higher Pb concentration.

### 3.2 Microbial biomass carbon variation in different organic substrate ratios

The interaction effect (tailings source  $\times$  organic substrate rate) was not significant (Table 1) for the microbial biomass carbon (MBC), but both main factors were ( $P \leq 0.05$ ) (Results shown in Supplementary material, Fig. S1a). The MBC was about 1.5-fold higher in tailings B than tailings A. Compared with the non-amended tailings (CM<sub>0</sub>), the MBC was 19-fold higher in CM<sub>75</sub> (Results shown in Supplementary material, Fig. S1b).

The interaction effect (tailings source  $\times$  organic substrate rate) was not significant (Table 1) for microbial quotient (MBC/TOC), but the organic substrate rate was a significant main factor (see Supplementary material, Fig. S2). The lowest microbial quotient was in 0% organic substrate, and the highest was in 100% organic substrate rate. The results showed an increasing trend in microbial quotient with increasing organic substrate rate, with 50% and 75% organic substrate treatments showing, respectively, 17- and 33-fold higher microbial quotients than the control.

### 3.3 Incubation study to determine soil respiration

The repeated measures ANOVA showed a significant interaction of tailings source  $\times$  organic substrate rate  $\times$  incubation

days in cumulative respiration measurements ( $P \leq 0.05$ ) (Fig. 1a). Tailings A treatments followed the same pattern as tailings B treatments but with a lower rate of cumulative CO<sub>2</sub> emission, and the addition of organic substrate caused a significant increase in respiration (Fig. 1b).

Cumulative CO<sub>2</sub> emissions from the amended tailings treatments increased significantly after 12 days ( $P < 0.0001$ ) of incubation and tended to stabilise after 91 days ( $P < 0.0001$ ). Similarly to MBC (Fig. S1), the cumulative soil respiration was considerably higher in organic substrate (CM<sub>100</sub>) (Fig. 1a), followed by CM<sub>75</sub> and CM<sub>50</sub> in both tailings, with the non-amended tailings (A-CM<sub>0</sub> and B-CM<sub>0</sub>) being the lowest.

### 3.4 Plant root morphological traits

The total root length of perennial ryegrass grown in tailings A and B was not significantly affected by the interaction effect (Table 1), but the significant main effects (see Supplementary material, Fig. S3) of tailings source and organic substrate rate were observed ( $P \leq 0.05$ ) after 60 days of growth. The perennial ryegrass grown in tailings B exhibited about 2-fold greater root length compared to tailings A ( $P \leq 0.05$ ). The 75% organic substrate treatment resulted in 2-fold greater root length than unamended (0%) tailings.

A significant interactive effect of tailings source and organic substrate rate was observed on the average root diameter of perennial ryegrass after 60 days of growth (Table 1). The average root diameter was significantly larger in B-CM<sub>75</sub> (75% organic substrate rate) than in all the other treatments. The smallest average root diameter was reported in unamended tailings A, and there was no significant difference in average root diameter among the treatments A-CM<sub>75</sub>, B-CM<sub>0</sub>, B-CM<sub>50</sub>, and CM<sub>100</sub>.

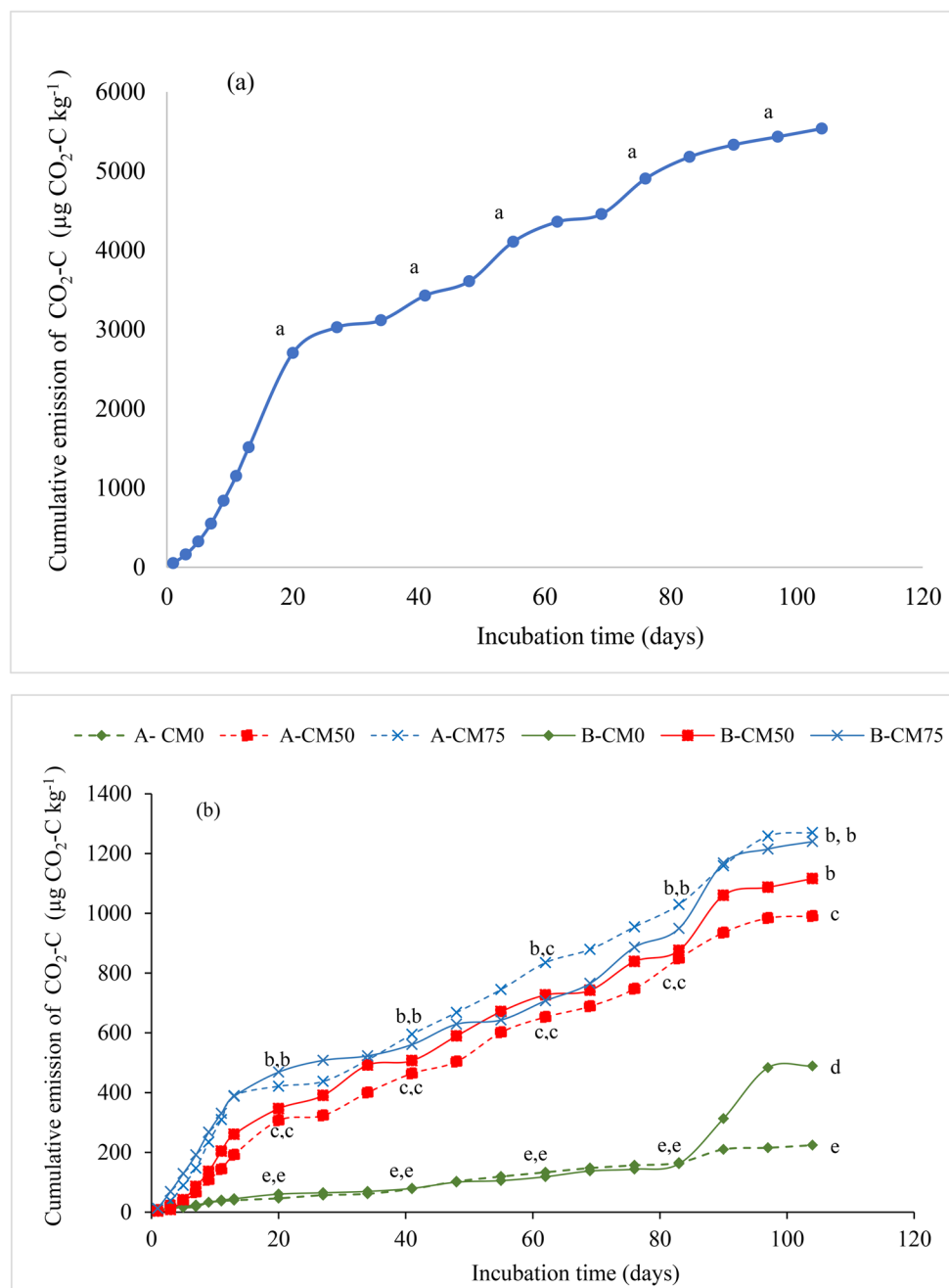
The interaction (tailings source and organic substrate rate) significantly ( $P \leq 0.05$ ) influenced root dry biomass of perennial ryegrass grown for 60 days (Table 1). About

**Table 1** Effect of different compost application rates (w/w %) on tailings A and B at harvest 2, after 60 days of *Lolium perenne* growth on microbial biomass carbon ( $\mu\text{g C g}^{-1}$ ), microbial quotient (MBC/TOC), mycorrhizae colonisation (%), root length (m pot<sup>-1</sup>), root diameter (mm pot<sup>-1</sup>) and root dry biomass (g pot<sup>-1</sup>)

| Treatment         | MBC           | MBC/TOC            | Mycorrhizal colonisation | Root length        | Average root diameter | Root dry biomass  |
|-------------------|---------------|--------------------|--------------------------|--------------------|-----------------------|-------------------|
| ACM <sub>0</sub>  | 3 $\pm$ 0.1d  | 0.36 $\pm$ 0.24d   | 0.06 $\pm$ 0.01f         | 1.36 $\pm$ 0.01c   | 0.01 $\pm$ 0.00e      | 0.1 $\pm$ 0.00d   |
| ACM <sub>50</sub> | 42 $\pm$ 3 cd | 3.92 $\pm$ 1.22 cd | 1.4 $\pm$ 0.01e          | 7.13 $\pm$ 0.77c   | 0.1 $\pm$ 0.01d       | 0.3 $\pm$ 0.05d   |
| ACM <sub>75</sub> | 102 $\pm$ 5bc | 12.16 $\pm$ 1.05ab | 3.6 $\pm$ 0.1c           | 24.71 $\pm$ 3.42bc | 0.2 $\pm$ 0.02bc      | 1.5 $\pm$ 0.12c   |
| BCM <sub>0</sub>  | 9 $\pm$ 7d    | 0.29 $\pm$ 0.23d   | 0.06 $\pm$ 0.01f         | 43.41 $\pm$ 9.02ab | 0.3 $\pm$ 0.03bc      | 1.7 $\pm$ 0.13c   |
| BCM <sub>50</sub> | 135 $\pm$ 43b | 7.41 $\pm$ 2.64bc  | 2.3 $\pm$ 0.2d           | 45.91 $\pm$ 9.60ab | 0.2 $\pm$ 0.02b       | 2.7 $\pm$ 0.02ab  |
| BCM <sub>75</sub> | 174 $\pm$ 7ab | 7.66 $\pm$ 0.39bc  | 5.5 $\pm$ 0.14b          | 55.52 $\pm$ 12.12a | 0.5 $\pm$ 0.08a       | 2.8 $\pm$ 0.02a   |
| CM <sub>100</sub> | 217 $\pm$ 22a | 16.08 $\pm$ 0.31a  | 7.8 $\pm$ 0.15a          | 47.90 $\pm$ 2.16ab | 0.3 $\pm$ 0.04b       | 2.40 $\pm$ 0.01ab |
| S                 | 0.028         | 0.785              | 0.000                    | 0.000              | 0.000                 | 0.000             |
| R                 | 0.000         | 0.000              | 0.000                    | 0.042              | 0.030                 | 0.000             |
| S $\times$ R      | 0.194         | 0.071              | 0.000                    | 0.151              | 0.032                 | 0.000             |

S= tailings source, R= organic substrate rate, w/w %. Different letters in a column indicate significant differences at  $P \leq 0.05$  according to the Tukey HSD test

**Fig. 1** Effect of organic substrate only (**a**) and tailings A and B supplemented with different rates of organic substrate (**b**) on cumulative  $\text{CO}_2$  respiration during the 105-day incubation period



2-fold greater root dry biomass was recorded in B-CM<sub>50</sub> and B-CM<sub>75</sub> compared to the unamended tailings (B-CM<sub>0</sub>). A similar trend was observed in tailings A [A-CM<sub>50</sub> and A-CM<sub>75</sub> showed 29-fold and 150-fold, respectively, greater root dry biomass than unamended tailings (A-CM<sub>0</sub>)]. Interestingly, the treatment CM<sub>100</sub> had lower (0.8-fold) root dry biomass than B-CM<sub>75</sub>.

### 3.5 Arbuscular mycorrhizal colonisation

The interaction (tailings source and organic substrate rate) was significant after 60 days of harvest (Table 1). After 60

days of perennial ryegrass growth, the mycorrhizal colonisation was 25- and 53-fold greater in tailings amended with 50% and 75% organic substrate, respectively, compared with the unamended tailings.

### 3.6 Principal component analysis (PCA)

The first two principal component axes explained 68% and 26% of the total variance (Fig. 2). The PCA results corroborated the similar patterns of root dry weight, total root length at 60 days harvest and physicochemical attributes: pH, TOC, CEC and DTPA-metal concentration in the substrate

(Ni, Co, Pb, Cd, Mn, Cr, Fe, Cu) and cumulative respiration. The MBC, CEC, TOC, cumulative respiration, and DTPA-metal concentrations (e.g., Cd) appeared closely related to the second PCA axis (Fig. 2). Most substrate physico-chemical parameters overlapped with A-CM75, B-CM50, B-CM75, and CM<sub>100</sub>. The root dry weight, MBC, respiration, TOC and DTPA metals (except Co, Cr and Ni) were significantly associated with those treatments. However, pH and EC did not contribute to any of the treatments in this study, and it further showed that A-CM<sub>0</sub> and B-CM<sub>0</sub> are distinctly different.

### 3.7 Influence of treatments on bacterial community composition

The interaction (tailings source  $\times$  organic substrate rate) significantly influenced the relative abundance of the phyla Bacteroidetes and Gemmatimonadetes, whereas Actinobacteria, Proteobacteria, Chloroflexi, and others were influenced by the main effect of organic substrate rate. The relative abundance of the phylum Verrucomicrobia was significantly influenced only by the main effect of the tailings source.

The phyla Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes were abundant in our study (Fig. 3), with the relative abundance of Proteobacteria exceeding 20% in

all the treatments (Fig. 3). Interestingly, the treatment without organic substrate (both A-CM<sub>0</sub> and B-CM<sub>0</sub>) showed a higher abundance ( $P \leq 0.05$ ) of Proteobacteria than the other treatments. In the 50% organic substrate treatment, the relative abundance of Proteobacteria did not differ significantly between the two types of tailings.

The relative abundance of Actinobacteria was lower in the unamended tailings (Fig. 3) and increased with increasing organic substrate rate. This increase was greater in tailings A than tailings B.

In the phylum Bacteroidetes, the unamended tailings had a higher (~2-fold) relative abundance than the treatments containing organic substrate. Furthermore, tailings B had a significantly lower relative abundance than tailings A, with the difference increasing proportionally with the organic substrate rate.

### 3.8 Alpha diversity and beta diversity

Bacterial community diversity within the tailings was evaluated using the Shannon index and OTU richness, both of which were significantly affected by the treatment interaction (Fig. 4a). Overall, the organic substrate-amended tailings exhibited significantly higher Shannon diversity and OTU richness than the unamended control ( $P \leq 0.05$ ), with

**Fig. 2** Principal component analysis of treatments and measured variables. Root length, root dry weight, shoot dry weight, CEC, MBC, CO<sub>2</sub> respiration, pH, EC, TOC and DTPA-extractable metals (Cu, Mn, Cd, Zn, Co, Pb, Fe, Ni, Cr) in iron ore tailings A and B treated with various rates of organic substrate and supporting *Lolium perenne* growth for 60 days

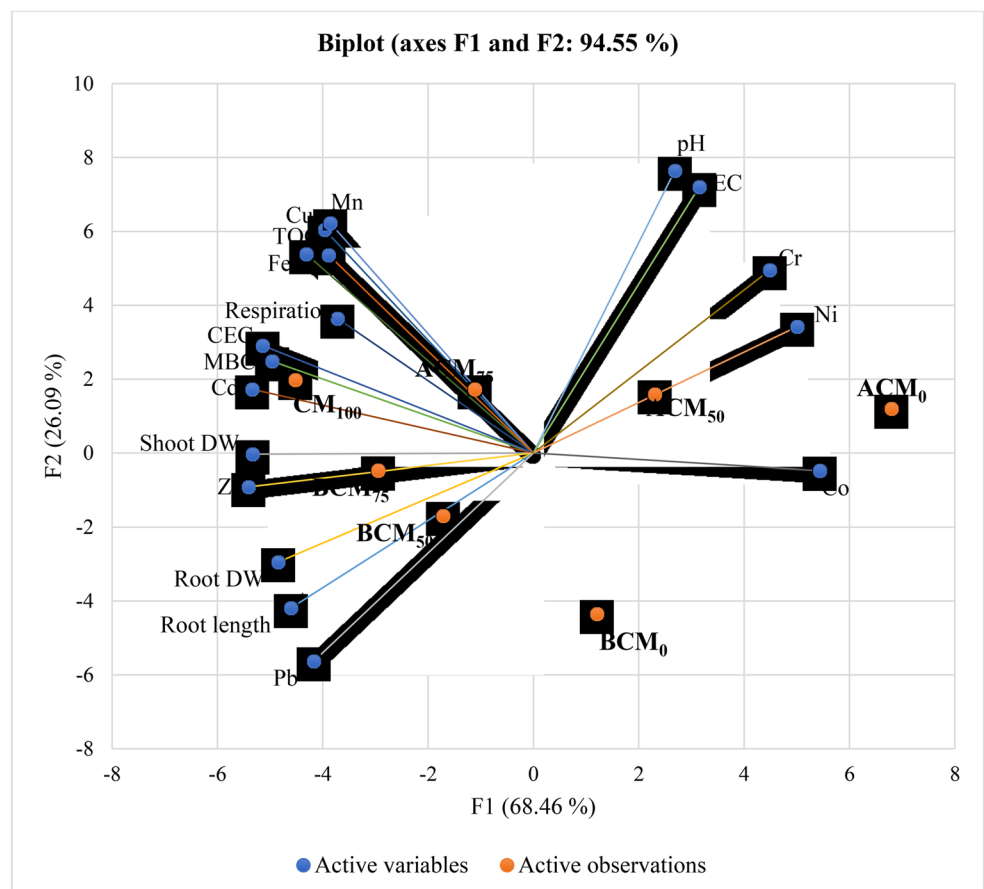
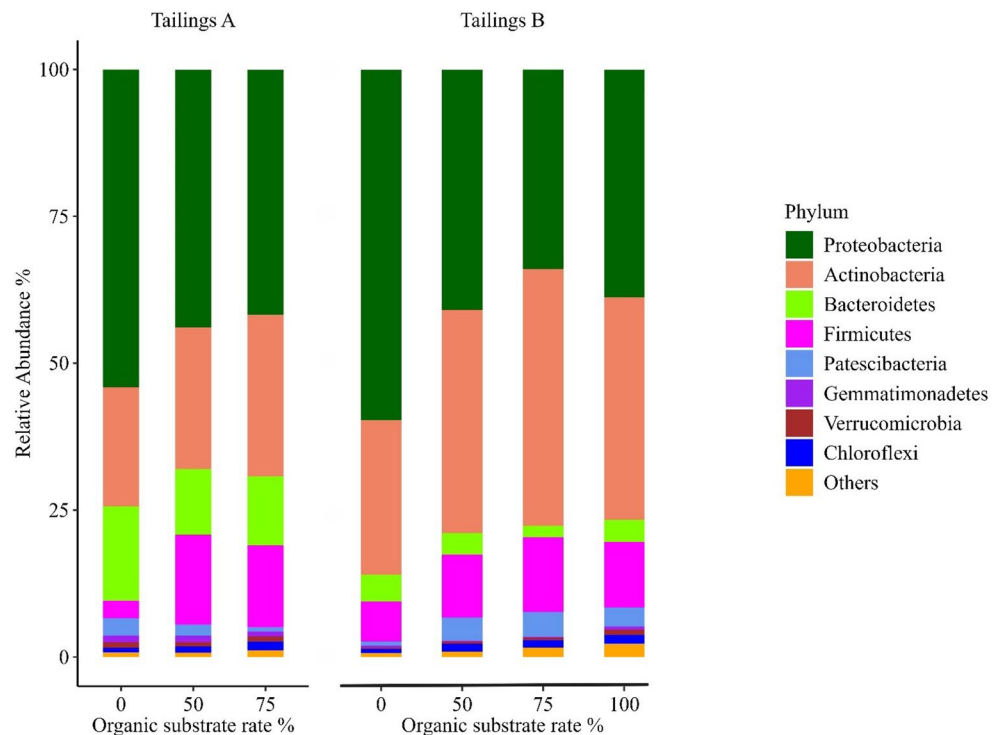


**Fig. 3** Effects of different organic substrate rates (% w/w) on the composition of the bacterial community (at the phylum level) in the rhizosphere of *Lolium perenne* grown in tailings A and B for 60 days. The bacterial phyla with a relative abundance of <1% are shown as “others”



the exception of the 75% w/w organic substrate treatment in tailings B, where no significant increase was observed. Non-metric multidimensional scaling (NMDS) based on Bray–Curtis’s dissimilarities revealed clear differences in microbial community composition among the treatments (Fig. 4b). The unamended tailings (0%) formed a distinct cluster near the x-axis, indicating a community structure markedly different from that of all amended treatments. In contrast, the 50% and 75% organic substrate amendments showed the greatest separation from 0%, demonstrating that these amendment levels produced microbial communities most compositionally distinct from the unamended tailings. The 100% organic substrate treatment showed a shift to the positive, suggesting a highly pronounced community restructuring at the highest amendment level. Tailings B exhibited stronger treatment-driven separation than tailings A, indicating a greater responsiveness of its microbial community to organic substrate incorporation. The NMDS stress value of 0.104 indicates a good ordination fit, confirming that the two-dimensional configuration reliably represents the underlying community dissimilarities.

### 3.9 Canonical correspondence analysis (CCA)

The results of the CCA were significant ( $P \leq 0.05$ ), and the first axis explained 76% of the variance (Fig. 5). The positive side of the CCA 1 axis was mainly defined by increasing values of root length (RL) and substrate properties of total organic carbon (TOC) and microbial biomass carbon

(MBC). On the other hand, the positive side of the CCA 2 axis was mainly defined by high pH and EC values ( $r = 1.0$  and  $1.2$ ), and the negative side was mainly conditioned by lower values of these parameters; hence, a gradient was generated along the CCA 2 axis that segregated the samples from unamended treatments and tailings source (A and B): majority of the samples from tailings A were depicted on the negative side of the CCA 2 axis, whereas corresponding tailings B samples appeared on the positive side of the CCA 1 axis. The phyla Actinobacteria, Firmicutes, Chloroflexi, and Patescibacteria increased towards TOC, MBC, and RL, while Verrucomicrobia were mostly associated with pH and EC. However, Proteobacteria did not respond to any of the physicochemical parameters.

### 3.10 Changes in abundance of N- and C-cycling genes in response to soil organic substrate rate and tailings source

Based on the predicted rhizosphere bacterial functional genes, the most abundant C-cycling gene was *SrpA* (catalase, involved in decomposition of lignin) and the least abundant was *SusB* (glucoamylase, involved in amylose decomposition) (Fig. 6a). Adding organic substrate at any rate to tailings A significantly increased the abundance of C-cycling genes, except *SusB* (glucoamylase).

However, the *SrpA* gene appeared to be the most abundant predicted gene ( $n = 8$ ) in unamended tailings B than in other treatments, whereas *bglX* and *bcsZ* were significantly

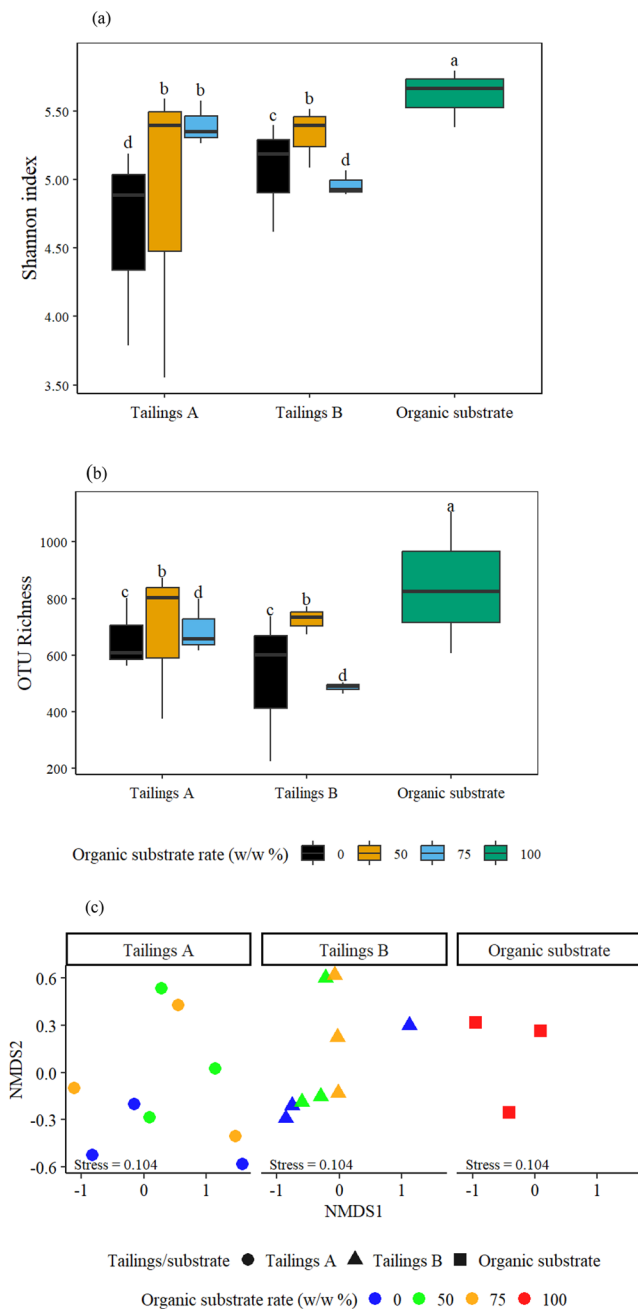


**Fig. 4** The comparison of bacterial alpha diversity [(a) Shannon index and (b) OTU richness] and beta diversity [(c) Non-metric Multidimensional Scaling (NMDS)] in the rhizosphere of *Lolium perenne* grown for 60 days in tailings A and B amended with various rates of organic substrate. Means  $\pm$  s.e.,  $n=3$ . In each graph, different letters indicate significant differences at  $P \leq 0.05$ , according to the Tukey HSD test

more abundant in 75% organic substrate application than in other treatments. Interestingly, the *lacZ* level was significantly lower with 75% organic substrate than with 50% organic substrate. Predicted abundance of C genes such as *amyA* and *SusB* showed the lowest abundance among other genes. Notably, the abundance of C-cycling genes was

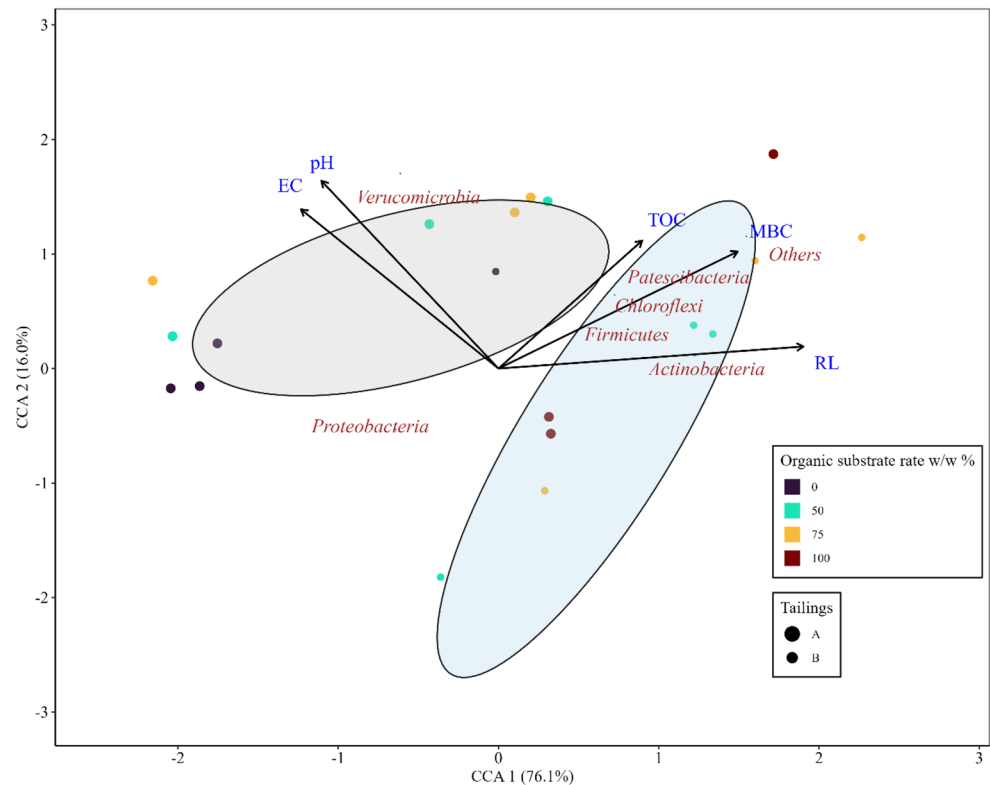
enhanced with increasing organic substrate application rate in tailings B (Fig. 6a).

Functional genes relating to N and C cycling in the system were selected based on PICRUSt predictions. Overall, a high proportion of denitrification genes were found in all the treatments in both tailings A and tailings B (especially *narG* and *nirK*), followed by N-fixation genes (*nifD*) and least abundant in nitrification (*amoA.amoB* and *Hao*) (Fig. 6b). In the denitrification process, *narG* gene abundance was significantly higher (27%) ( $P \leq 0.05$ ) in 100% organic substrate than 0% organic substrate (control) in tailings A, whereas in tailings B *narG* was highest in 0% organic substrate (31%). Relative abundance of the *nosZ* gene was significantly higher in 50% and 75% than in 100% organic substrate in tailings A, but not with 0% organic substrate treatment. However, in tailings B, the *nosZ* gene showed a significantly higher abundance in the 0% organic substrate treatment than in the other treatments. Relative abundance of the *nrfA* gene was not significantly different among the treatments in both tailings A and B (Fig. 6b). In the nitrification process, abundance of both *amoA.amoB* and *Hao* genes was not significantly different either among treatments or tailing sources. Regarding the N fixation process, there was no significant difference in *nifD* relative abundance among all treatments in tailings A, whereas 50% organic substrate treatment in tailings B showed a significant difference among the treatments (Fig. 6b).

## 4 Discussion

DTPA-extractable heavy metals, which have known available forms and primarily exist in water-soluble and ion-exchangeable states, can be directly absorbed by plants (Chen et al. 2019). It is considered that the metal fraction extracted with chelating agent DTPA can be considered as potentially available to plants (Singh and Kalamdhad 2015). The response of DTPA-extractable metal concentrations (Zn, Cu, Fe, and Mn) to the increasing rate of organic substrate addition was generally linear throughout the experiment. This could be due to the high bioavailable metal content in the organic substrate by solubilisation and complexation (Maqueda et al. 2010) and the application of organic substrate may change the tailing's interior environment (Chen et al. 2019) by increasing the dissolution of organic matter during the DTPA extraction procedure (Singh and Kalamdhad 2015). However, DTPA-extractable Cr was reported to decrease proportionately with increasing rate of organic substrate due to the affinity of Cr for entering organic complexes (Kumar et al. 2025; Singh and Kalamdhad 2015), or this is probably due to incomplete extraction or low mobility and availability of Cr in an insoluble form (Walter et al. 2006), reduction from Cr(VI) to Cr(III) (Kumar et al. 2025) or strongly binding to the primary minerals (Vega et al. 2004).

**Fig. 5** Canonical correspondence analysis (CCA) biplot showing the relationship between tailings source, organic substrate rate, and measured physicochemical variables of tailings (pH, EC, TOC and MBC) with root length (RL) after 60 days of *Lolium perenne* growth



The increase in microbial biomass in this long-term experiment with organic amendments is mainly due to the microbial biomass contained in the organic residues and the addition of substrate-C, which stimulates the indigenous soil microbiota, as confirmed by 100% organic substrate analyses (García-Gil et al. 2000). Organic substrate application may have increased microaggregate formation by sustaining higher bacterial populations across aggregate fractions (Wang et al. 2022a). Therefore, organic substrate addition can potentially generate a positive feedback loop that enhances soil C storage by increasing microbially available substrate, thereby increasing and maintaining soil microbial biomass and, in turn, increasing aggregation through microbial activities (Wang et al. 2022a). Moreover, organic substrate application can indirectly increase nutrient availability by increasing microbial activity and abundance (Verma and Marschner 2013).

Organic substrate additions to soil can have negative, neutral, or positive effects on the colonisation of roots by AM fungi, which is highly dependent on organic substrate maturation and parent material (Mickan et al. 2017). However, in this study, the addition of an organic substrate increased AM fungal colonisation, benefiting root growth and thus plant performance/resilience, either directly or indirectly under stress conditions, such as nutrient limitation and metal toxicity. Few studies reported that a combination of organic substrate addition and arbuscular mycorrhizal inoculation produced positive and synergistic

effects on the phytomanagement of a semiarid mine tailings (Kohler et al. 2015) and a possible mechanism for engaging in the remediation process is that production, release and accumulation of glomalin-related soil protein (GRSP) generate complexes within the entity, but the active mechanism of GRSP in chelating heavy metals further requires research (Ai et al. 2020) and also altering rhizospheric pH and redox conditions, thereby reducing metal bioavailability and toxicity to the host plant (Chen et al. 2025). Extensive extraradical hyphal networks, AM fungi increase the effective root surface area, allowing plants to access phosphorus, nitrogen and micronutrients (Wang et al. 2022b) that are otherwise immobile or located in microsites beyond the root (Khoza et al. 2025). This multifunctional ability of AM fungi enhances nutrient acquisition while immobilising metals-supporting plant growth and survival in nutrient-limited or metal-contaminated environments (Chaudhary et al. 2025) tailings.

Organic amendments, such as organic substrate, are useful tools in the recovery of the function and structure of the microbial community in soils contaminated with metals (Pardo et al. 2011). In the present study, organic substrate provoked both mine tailings by increasing CO<sub>2</sub> production/respiration, indicating that the degradation of part of the organic matter is provided by the addition of organic substrate, especially more slowly mineralisable compounds (Pardo et al. 2011), and needs more incubation time to enhance microbial activity at a reliable rate.

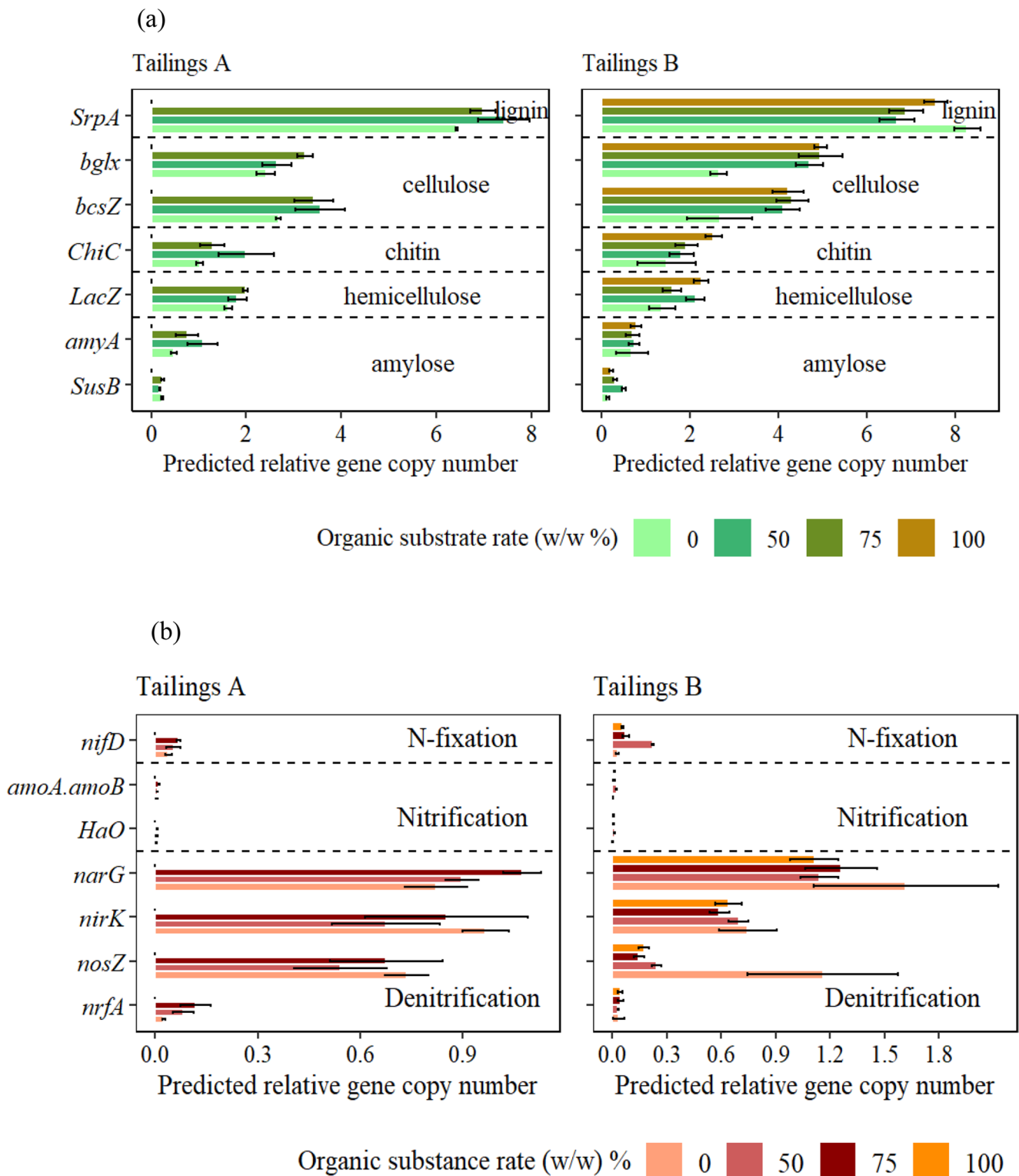


**Fig. 6** Predicted relative functional gene copy numbers ( $10^{-2}$ ) (PIC-RUST) associated with carbon cycling (a) nitrogen cycling genes (b) in the bacterial community of the rhizosphere of perennial ryegrass, as influenced by the organic substrate treatment (0, 50, 75, 100%, w/w) and tailings source (A and B). The C-cycling genes encoding specific enzymes are: *SrpA* (catalase), *bglx* (beta-glucosidase), *bcsZ* (endoglucanase), *ChiC* (chitinase), *LacZ* (beta-galactosidase), *amyA* (alpha-amylase), and *SusB* (glucoamylase). The following N-cycling

genes encoding specific enzymes are included: nitrogen-fixing gene *nifD* (nitrogenase); nitrifying genes *amoA/amoB* (ammonia monooxygenase) and *Hao* (hydroxylamine oxidoreductase), and denitrifying genes *narG* (nitrate reductase), *nirK* (nitrite reductase), *nosZ* (nitrous oxide reductase) and *nrfA* (cytochrome c nitrite reductase–ammonium forming). The substrates for the tested enzymes are specified in the graphs. Means  $\pm$  s.e.,  $n=3$

In general, the dominant bacterial phyla in mining areas include Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes (Li et al. 2021), which can alleviate the hazardous toxicity of metals via specialised mechanisms (Fashola et al. 2016). Proteobacteria encompasses many facultative anaerobic members and is always present as the most abundant phylum in several non-ferrous mine tailings areas and iron mine areas in Beijing (Li et al. 2021). Previous studies have observed shifts in bacterial composition following short- and long-term soil amendment with an organic substrate (Mickan et al. 2017), findings consistent with ours. Furthermore, there was greater bacterial community richness and diversity in the rhizosphere of the mine tailings following the addition of an organic substrate, as previously reported (Zhen et al. 2014). This can occur due to increased population size and heterotrophic bacterial activity as labile C compounds enter the tailings. However, these changes are attributed to an allochthonous bacterial community introduced from the organic substrate to the tailings. Regarding phylum abundance, organic substrate significantly increases the percentages of Actinobacteria and Firmicutes and decreases those of Proteobacteria and Bacteroidetes. This composition shift can be interpreted within the framework of microbial r/K selection theory, where r strategists (e.g., Many Proteobacteria) dominate under conditions of abundant labile carbon, while K-strategists (e.g., Actinobacteria) (Hu et al. 2023) are favoured as resources become limited and recalcitrant carbon (hemicellulose, cellulose, and chitin) predominates. Further to Hu et al. (2023), compared to r-strategists, which tend to use fresh exogenous organic matter, K-strategists grow slowly and mainly use inexhaustible soil organic matter. Moreover, K-strategists can degrade the non-readily oxidisable organic carbon to obtain nutrients required for their growth, thus gradually dominating, especially in the forest context (Fontaine et al. 2003).

Members of the fast-growing phyla are very adaptable and capable of degrading various organic compounds, thereby increasing the availability of essential micro- or macronutrients to plants. These phyla compete for available resources and exhibit the highest growth rates. Further, based on CCA analysis, the proliferation of these phyla (Actinobacteria, Firmicutes, Chloroflexi, Patescibacteria) was linked to an increase in TOC and MBC associated with organic substrate amendment. Perhaps the organic substrate creates a physical niche where bacteria can proliferate, especially given its high surface-area-to-volume ratio in mine tailings.

The addition of an organic substrate increased the predicted lignin and amylose-degrading functional genes (e.g., *SrpA*, *amyA* and *SusB*, respectively) and decreased cellulose, chitin, and hemicellulose (*bglX*, *bcsZ*, *ChiC* and *lacZ*) (O'Connor et al. 2024; Shanmugam et al. 2021). The most prominent gene associated with C cycling in this study was

*SrpA*, and the least was *SusB*. However, amending tailings with an organic substrate has previously been shown to increase hydrolytic enzyme activities involved in C-cycling (Mickan et al. 2017). An abundance of functional nitrogenase (*nifD*) genes was detected during nitrogen fixation, particularly with higher rates of organic substrate application. This is consistent with previous studies that found N-fixing compositions were more abundant and diverse in the clay soils due to better aggregation, and mine tailings used in this study showed a similar physical structure (Sarathchandra et al. 2022). However, nitrification was not a prominent process in either tailings and could not be stimulated by organic substrate application for several reasons. Nitrifiers are autotrophic organisms having extremely complex cell structures and are thus thought to be sensitive to minor changes in environmental conditions such as soil quality, and as several studies suggest, they inhibit nitrification due to metal contamination. As reported by Cela and Sumner (2006), Cu and Zn impaired nitrification, whereas Stuczynski et al. (2003) observed that Zn inhibited nitrification and several other enzymatic activities. Denitrifiers are heterotrophic organisms found within most taxonomic groups of bacteria and are suitable representatives of the entire bacterial soil community. They depend on the availability of carbon and energy, such as sugar and cellulose. Organic substrates supply organic carbon to the system, promoting the growth of denitrifiers. Organic substrate application will encourage the activity of aerobic (O<sub>2</sub>) microorganisms, whose respiration will eventually create a more anaerobic environment, such as in mine tailings. This could explain the higher abundance of *narG* in tailings B compared to tailings A after organic substrate application. Organic substrate application increases the episodic influx of bioavailable carbon, including both labile and recalcitrant fractions (as explained previously), which promotes heterotrophic microbial respiration and increases microbial activity in soil. This process augmented microbial respiration to rapidly diminish oxygen availability, creating transient anoxic or hypoxic microsites around the organic substrate particles (Lacroix et al. 2024) and within tailings particles. Under these conditions, microbes switch to alternative electron acceptors, with nitrate being reduced via the membrane-bound nitrate reductase encoded by the *narG* (Philippot et al. 2013). The elevated availability of organic carbon provides the reducing equivalents necessary for denitrification, enhancing the proliferation of *narG*-harboring microbe, such as certain Proteobacteria and Actinobacteria (Dai et al. 2018). Consequently, organic substrate-mediated processes increase in carbon availability and the formation of oxygen-limited microsites collectively explains the observed enrichment of *narG* in microbial communities.

However, in the broader context, PICRUSt2 also has some limitations itself as reported by Langille et al. (2013). PICRUSt predictions are based on the availability of reference genomes and may be potentially imprecise for environments such as mine tailings that contain novel or under-represented taxa in genome databases. Further to Langille et al. (2013), it is cautioned that PICRUSt2 predictions should ideally be validated using metagenomic or absolute functional data (for example, qPCR or enzyme assays).

## 5 Conclusion

Organic substrate application (75% w/w) to mine tailings has a positive impact on the root traits of perennial ryegrass, and on rhizospheric microbial activities, compared to unamended tailings. The relative abundance of specific microbial phyla increased with 75% organic substrate application, and functional genes associated with carbon cycling (lignin degradation) and nitrogen cycling (denitrification) were affected by organic substrate application. In this study, tailings B performed better than tailings A due to better physicochemical characteristics, including lower EC, higher TOC, and higher MBC. To comprehensively evaluate the effects of organic substrate application on microbial activities, biogeochemical cycling, and perennial ryegrass-related root morphological traits in iron ore mine tailings, long-term field trials are recommended to capture temporal variability and potential cumulative impacts, given the limited studies on iron ore mine tailings in Western Australia. Arguments can be made for the organic substrate proportion used in this glasshouse trial and the practicability of using organic substrate ratios in field conditions. However, the initial costs associated with tailings conditioning with organic substrate are primarily incurred during the early stages of rehabilitation, while subsequent expenditures are likely to be lower than the ongoing costs associated with other rehabilitation activities.

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**Data availability** The data generated in this study have been deposited in the GitHub Read Archive (SRA) under first author's name and will be made available on request.

## Declarations

**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.

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