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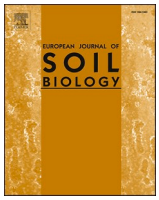

Original article

Effects of different soil organic amendments (OAs) on extracellular polymeric substances (EPS)

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ABSTRACT

Extracellular polymeric substances (EPS) synthesized by soil microorganisms play a crucial role in maintaining soil structure by acting as binding agents of soil aggregates. Microbial EPS production is governed by C sources, soil nutrient availability, pH, and other local environmental factors. Another important factor is soil management, and particularly, the addition of organic amendments (OAs), has the potential to influence soil EPS as it can change the biotic and abiotic properties of the soil. Yet the response of soil EPS to the addition of OAs, especially in field trials, and its subsequent impact on soil aggregation remains unclear. This study aimed to elucidate the influence of OAs (including compost from organic residues, mown grass from roadsides and parks, and cattle manure) on soil EPS content and aggregate stability in a three-year field experiment with annual OA application. We further investigated factors that govern EPS production in the soil by exploring the relationship between soil EPS (i.e., polysaccharide and protein content), soil physicochemical properties (i.e., pH, dissolved organic carbon, available and total amount of nutrients), and the soil microbial community (i.e., microbial abundance and taxonomic structure). We found that the addition of grass, manure, and the combination of grass and manure led to an increase in soil EPS content compared to unamended and compost-amended soils. EPS content was correlated with soil variables; in particular, a significant positive correlation was observed between EPS concentration and available N in the soil. Furthermore, bacterial and fungal biomass contributed to soil EPS. Specific bacteria (e.g., members of Proteobacteria, Bacteroidetes, and Chloroflexi) and fungi (e.g., members of Ascomycota and Basidiomycota) demonstrated strong and significant correlations with EPS in the soil. The direction of correlation, whether positive or negative, varied at the order level. In addition, our study revealed significant positive correlations between EPS concentration and soil aggregate stability. These findings offer insights into designing sustainable agricultural management practices, and whether the application of appropriate OAs can enhance soil EPS content and, consequently, soil aggregate stability.

1. Introduction

In their natural environment, microbes are predominantly associated with surfaces [1]. During the sessile growth mode, microbes produce extracellular polymeric substances (EPS), which are highly hydrated and charged [1,2]. While the composition of EPS can vary greatly, polysaccharides and proteins are considered to be the major fractions of EPS [3]. In the soil, EPS are intermixed with cells and other soil organics and minerals, providing diverse benefits to microbes. EPS can affect soil functioning, as they are viewed as highly responsive transient binding agents. EPS normally do not persist in the soil; instead, they have a short turnover time. Research shows that they can be more affected by current soil management than by legacy effects from previous management, even when applied continuously for a duration of over 50 years [4,5].

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EPS can facilitate microaggregate formation, increase water holding capacity, and improve soil structural stability more effectively than bulk soil organic matter (SOM) [4,5]. This benefits agriculture, especially in environments that are dry and with deficit irrigation [6,7]. EPS act as sponges and retain water, delay drying, and connect microorganisms with substrates, thereby supporting microbial activity even at low water potential [7–9]. Due to the wide range of benefits that EPS bring to the soil, the management of soil EPS is gaining increasing interest in agricultural practices. Studies have been conducted to understand EPS dynamics influenced by both previous and current land use, where they have investigated the effects of agricultural management practices, such as soil water management and organic input, on EPS and soil aggregation [5,10–12].

Microbial EPS production can be promoted by changing environmental variables (e.g., soil pH, nutrient availability, carbon-to-nitrogen (C:N) ratio, and water content) [13–15] or by introducing/stimulating EPS-producing pure cultures [16,17]. The addition of organic amendments (OAs) is another potential way of promoting EPS production in the soil since OAs are rich in C substrates and nutrients, yet this approach has received less attention than the addition of pure cultures, and only a few studies have investigated EPS in field trials so far [5,10,18]. It is currently unclear to what extent OAs can enhance soil EPS and aggregate stability and which soil microorganisms are potentially responsible for building the soil EPS matrix in response to the addition of OAs. Previous studies have found that soil EPS content is positively correlated with microbial biomass [9,19]. In addition, The availability of C substrates stimulates microbial EPS production and altering soil N levels also regulates EPS content [10,20,21]. The availability of different carbon sources can directly affect microbial EPS production by influencing the precursor molecules necessary for EPS synthesis [12,22]. The availability of N can influence the composition of soil EPS and the quantity of polysaccharides produced by impacting the N metabolism of the microbial community [23]. Sher et al. [12] found that soil EPS can be influenced by root biomass, which potentially regulates fresh C supply. Hale et al. [10] reported enhancements in soil microbial biomass, soil EPS, and soil aggregate stability with the addition of compost.

To understand the influence of key physicochemical and biological variables on EPS content in agricultural soil, we conducted a three-year field experiment. We tested the effects of different OAs, including compost in high and low doses, mown grass, cattle manure, and a mixture of mown grass and cattle manure, on soil EPS concentration and aggregate stability. Compost, grass, and manure are commonly used in agriculture as soil amendments to increase SOM and nutrients [24,25]. These OAs vary in nutrient availability, C stability (i.e., bioavailability and biodegradability of OM), and microbial activity [26], potentially affecting soil EPS content [10,12]. We expected that soils amended with OAs would have higher EPS concentrations than unamended soil. Specifically, we expected that the addition of grass, manure, and their combination, providing more labile organic C and nutrients for microbial growth, would result in a higher absolute quantity of EPS compared to compost addition. Additionally, since grass typically contains higher fractions of lignin and cellulose than manure, and manure exhibits higher biological activity than grass [26,27], we anticipated that the addition of manure would provide greater support for microbial growth and consequently, EPS production in the soil compared to grass. Furthermore, we hypothesized that the addition of OAs would induce changes in the soil microbial community. Specifically, we expected that the addition of OAs would increase microbial biomass and alpha diversity compared to the control due to the provision of C sources and nutrients. We anticipated that soil EPS concentration would correlate with specific microbial taxa, potentially contributing to EPS production or degradation in the soil. By testing these hypotheses, we aim to identify key physicochemical and biological variables that correlate with EPS content and soil aggregation in agricultural soil.

2. Material and methods

2.1. Field experiment description and soil sample collection

The experimental field is located near Heelsum, The Netherlands. The soil is classified as a coarse sandy Anthrosol (WRB-FAO classification), comprising 74 % sand, 20 % silt, 2 % clay, and 3.7 % organic matter. Maize (*Zea mays* L.) and *Lolium multiflorum Lam.* were grown in rotation, with the latter acting as a winter catch crop and being grown during the sampling season (February 2021). Six treatments were tested, including unamended soil (control, only mineral fertilizers were added), low-dose compost from organic residue (CL, ~11 ton/ha per year), high-dose compost of the same type (CH, ~22 ton/ha per year), mown grass from roadsides and parks (Gra, ~20 ton/ha per year), cattle manure (Man, ~30 ton/ha per year), and a combination of mown grass and cattle manure (Gra + Man, ~50 ton/ha per year). The combination represents a common practice in Dutch agronomic reality, where excess manure from the livestock industry is often combined with organic residues for soil amendments. The application rates varied yearly based on the quality of OAs and were designed in accordance with national fertilization recommendations and standard application norms [28,29]. We limited the total nutrient input to 120 kg/ha of available N, 50 kg/ha of P_2O_5, and 200 kg/ha of K_2O. Once one nutrient limit was met, deficits in other nutrients were compensated by adding mineral fertilizers, ensuring the total input of nutrients from OAs and mineral fertilizers met the maximum allowable input (Table 1) [24,25]. We had deviations in the inputs of OAs between years, which we considered acceptable, as they reflected the natural variation in OAs that one can expect in reality. The OAs were mechanically incorporated into the soil using a disc harrow to a depth of approximately 15 cm. The treatments were applied to 10 × 10 m plots in a randomized complete block design across a 30 × 60 m experimental field, as shown in Fig. 1. Each treatment was replicated three times, totalling 18 plots. The OAs were applied yearly from March 2018 until March 2020.

Five soil cores (10–20 cm deep, 3 cm diameter) were collected at random points from each plot in February 2021, 11 months after the last application round of the OAs. The soil samples were homogenized per plot and transported to the laboratory on ice. Upon arrival, soil samples from each plot were divided into four subsamples for physicochemical characterization, microbial composition analysis, and EPS visualization and quantification. One subsample was immediately fixed with 3 % paraformaldehyde buffered with phosphate-buffered saline (PBS 1X) to prevent cell lysis and to maintain its integrity for later microscopy visualization [30]. One subsample was immediately stored at 4 °C for water content (WC) and organic matter content (OM) analysis the day after. One subsample was immediately stored at −20 °C for DNA extraction which was carried out about one week later. The remaining subsample was immediately dried at 65 °C for three days until a constant weight was achieved for physicochemical characterization.

For the analysis of aggregate stability, a different sampling strategy was used. Changes in aggregate stability are subject to significant temporal variability. Yet we do not have the right tools to predict when the effect of OAs on aggregate stability is greatest (i.e., when it peaks). Since we did not want to miss the effect of OAs on aggregate stability by sampling too soon or too late, we decided to sample for aggregate stability twice per year (in 2019 and 2020), once in late June (three months after sowing) and once in late November (three months after harvesting/ploughing). By having sampled for aggregate stability multiple times within a year, we aimed to increase the robustness of our study and add more certainty to our conclusions.

2.2. Physicochemical analysis of soil

The WC of the soil was measured after drying in a forced-air oven at 105 °C for 8 h, and samples were subsequently burned at 550 °C for 2 h to quantify OM content. For dried soil samples, various parameters were

Table 1
An overview of the nutrient content in organic amendments (OAs) and the application dose of OAs and mineral fertilizers [24]. OM: organic matter; TN: total nitrogen; AN: available nitrogen. Control: unamended soil; CL: compost amended soil with low dose; CH: compost amended soil with high dose; Gra: mown grass amended soil; Man: cattle manure amended soil; Gra + Man: a combination of grass and manure amended soil.

Year	Treatment	Dose	Inputs from OAs kg/ha					Mineral fertilizer kg/ha		
		ton/ha	OM	TN	AN	P_2O_5	K_2O	N	P_2O_5	K_2O
March 2018	Control	0	0	0	0	0	0	140	50	200
	CL	10.6	1657	67	7	48	79	133	2	121
	CH	21.3	3329	136	14	96	158	126	0	42
	Gra	26.3	3570	373	186	123	208	0	0	0
	Man	34	1476	124	75	45	184	65	5	16
	Gra+Man	60.3	5046	497	261	168	392	0	0	0
March 2019	Control	0	0	0	0	0	0	106	65	200
	CL	12.3	1713	74	7	64	85	98	0	115
	CH	22.7	3174	138	14	118	157	92	0	43
	Gra	18.8	978	99	49	43	119	56	22	152
	Man	30	1386	128	58	42	141	47	23	59
	Gra+Man	48.8	2364	227	107	85	260	0	0	0
March 2020	Control	0	0	0	0	0	0	120	50	200
	CL	10.6	1593	71	7	39	57	122	11	143
	CH	21.8	3266	146	15	80	118	99	0	82
	Gra	13.5	2026	151	90	37	117	35	13	83
	Man	27.5	1386	114	54	40	113	67	10	87
	Gra+Man	41	3412	265	144	77	230	0	0	0

Fig. 1. Treatment arrangements in the field, located in Heelsum, The Netherlands. The four coordinates are: Top-right: 51°58′43.47″N, 5°45′59.06″E, Top-left: 51°58′43.57″N, 5°45′57.39″E, Bottom-right: 51°58′41.50″, 5°45′58.45″E, Bottom-left: 51°58′41.60″N, 5°45′56.85″E. CL: compost amended soil with low dose; CH: compost amended soil with high dose; Gra: mown grass amended soil; Man: cattle manure amended soil; Gra + Man: a combination of grass and manure amended soil.

assessed following the methods presented in the book "Soil Sampling and Methods of Analysis" [31] with modifications [32]. Given that water serves as the solvent and transport medium of nutrients for microorganisms and plants in the soil, we opted to use water, rather than alternatives such as $CaCl_2$, for nutrient extraction to better represent this reality. Specifically, these parameters include pH, electrical conductivity (EC), water-available nutrients (NO_3^-, NO_2^-, NH_4^+, PO_4^{3-}, K^+), total nutrients (including total carbon TC, total nitrogen TN, total phosphorus TP, and total potassium TK), and dissolved organic carbon (DOC). Soil pH and EC were measured using a Mettler Toledo SevenExcellence™ in a 1:10 soil/MilliQ water suspension (w/v) following 1 h of shaking at 25 °C. Soil WC was determined after drying in a forced-air oven at 105 °C for 4 h, with subsequent ignition at 550 °C for 2 h to quantify OM. TC and TN were determined using an elemental analyser (Interscience FlashSmart CHNSO). For TP and TK analysis, inductive coupled plasma optical emission spectrometry (PerkinElmer Optima 5300 DV) was employed after microwave acid digestion (Milestone Ethos Easy SK-15). Water-soluble nutrients (NO_3^-, NO_2^-, NH_4^+, K^+, and PO_4^{3-}) were measured in a 1:10 soil/MilliQ water suspension (w/v). The suspension underwent centrifugation at 3750*g* after 2 h of shaking at 25 °C. The supernatant, filtered through a 0.45 μm membrane filter (Hydrophilic PTFE), was then analysed using ion chromatography (Metrohm Compact IC 761). Additionally, DOC was extracted and prepared similarly to available nutrients and subsequently analysed using a TOC analyzer (Shimadzu TOC-L).

2.3. EPS quantification and visualization

Soil EPS were extracted using cation-exchange resins (CER) following the method described by Redmile-Gordon et al. [33]. First, 3 g of fresh soil was suspended in a 25 mL soluble microbial products (SMP) extraction solution. The solution consisted of 0.01 M $CaCl_2$ (local rainwater ionic equivalent) with pH 7 adjusted by 0.01 M $Ca(OH)_2$. The mixture of soil and the SMP extraction solution was shaken at 4 °C for 30 min at a speed of 2 cycles s^{-1}. After shaking, the supernatant containing SMP was discarded following centrifugation at 3200*g* for 30 min. Second, for EPS extraction, cation-exchange resin (CER, DOWEX Marathon C, sodium form) was pre-washed in phosphate-buffered saline (PBS 1X) twice. After pre-washing, CER was added to the centrifuged pellet along with 25 mL of chilled EPS extraction buffer. The amount of CER (g) was calculated using the equation: 2.543 x (SOC% x soil sample mass (g dry weight equivalent)) x 70. 2.543 is the conversion factor from carbon loss on ignition (LECO) to volatile solids (VS). The quantity of CER needed for EPS extraction is calculated based on the VS content in the soil, where sufficient CER equals 70 g of CER per gram of VS. The EPS extraction buffer contained 2 mM $Na_3PO_4{\cdot}12H_2O$, 4 mM $NaH_2PO_4{\cdot}H_2O$, 9 mM NaCl and 1 mM KCl with pH 7 adjusted by 1 M HCl, and cooled to 4 °C. The mixture of the centrifuge pellet and EPS extraction buffer was shaken at 4 °C for 2 h, with a speed of 2 cycles s^{-1}. The supernatant containing the extracted EPS was then centrifuged at 4000 *g* for 30 min, filtered to remove soil or plant residues, and further purified by dialysis.

The dialysis used tubular dialysis membranes with a 12–14 kDa molecular weight cut-off (Spectra/Por 2) against distilled water to remove low molecular weight metabolites and salts. The purified EPS solution was filtered with 0.45 μm filters (Hydrophilic PTFE) and then analysed using liquid chromatography-organic carbon detection (LC-OCD model 8, DOC-LABOR) to determine the amount of EPS carbon (EPS-C) and EPS nitrogen (EPS-N), representing polysaccharides and proteins, respectively.

Confocal laser scanning microscopy (CLSM) was employed to visualize EPS on soil particles. First, the paraformaldehyde-fixed soil samples were incubated at 4 °C overnight and then washed three times with Hanks' Balanced Salt Solution buffer (HBSS, ThermoFisher) before staining according to the method of Chen et al. [34]. Briefly, α-polysaccharides, cellulose, and DNA from living cells were stained with concanavalin A (Con A), calcofluor white (CW), and SYTO 63, respectively. SYTO 63 (20 μM), Con A (250 mg/L), and CW (300 mg/L) were sequentially added and incubated at 30 °C for 30 min after the addition of each dye. After each stage of the labelling process, the sample was washed three times with HSBB buffer to remove the excess dye. Before visualization, the labelled sample was embedded on glass slides and frozen at −20 °C.

2.4. Aggregate formation and stability

The geometric mean diameter (GMD) [mm] and mean weight diameter (MWD) [mm] of soil samples were determined using the wet sieving method [35], and as detailed by Kok et al. [25]. GMD reflects soil aggregate formation and the size of the aggregates, while MWD indicates the stability of soil aggregates [36]. Higher values of GMD and MWD indicate that soil aggregates contain more larger fractions and are also more stable. Briefly, soil samples were air-dried for two weeks and then oven-dried at 60 °C for 24 h before wet sieving. A sieve with a mesh width of 8 mm was initially used to remove roots, rocks and pebbles, as well as break large aggregates in the oven-dry samples. Subsequently, a sieve stack of descending sizes (2 mm, 1 mm, 500 μm, 250 μm, and 125 μm) was submerged into a column of water. Dry soil (20–40 g, the total mass was indicated as M_T) was slowly wetted with deionized water and then gently poured onto the top of the sieve stack. The sample was sieved under water in a vertical direction for 2 min at a frequency of 30 waves, of a 3 cm amplitude, per minute. Each sieve was then subsequently washed, and the dispersed aggregate (the mass of dispersed aggregate was indicated as M_{Ai}) and coarse material remaining (the mass of coarse was indicated as M_{Ci}) in each sieve were recovered and oven dried at 60 °C. GMD and MWD were calculated using the following equations:

$$\mathrm{GMD} = \exp\left[\frac{\sum_{i=1}^{n}(M_{Ai} * \ln(\mathrm{di}))}{M_T - \sum_{i=1}^{n} M_{Ci}}\right] \quad (1)$$

$$\mathrm{MWD} = \frac{\sum_{i=1}^{n}(M_{Ai} * \mathrm{di})}{M_T - \sum_{i=1}^{n} M_{Ci}} \quad (2)$$

Where $i = 1, 2, \ldots, n$ corresponds to each aggregate size fraction (n = 5), and *di* is the average diameter of each size fraction (i.e., mean inter-sieve size). It is important to note that we did not analyze the GMD and MWD indices for the soil receiving the low compost dose. It was physically not possible to sample all plots within a day for all the properties investigated at the field site. The decision was made to sample the high compost dose over the low compost dose given that we expected greater effect from the higher compost dose. Consequently, we excluded the EPS data of the soil receiving low compost dose from the correlation analysis with the GMD and MWD.

2.5. Characterization of bacteria and fungi

DNA was extracted from 0.25 g of soil using the DNeasy Power Soil Kit (Qiagen) following the manufacturer's protocol. DNA concentration and purity (OD_{260} and OD_{280}) were quantified using the Quant-it dsDNA kit on a Quantus (Promega) and the Nanodrop 1000 (Thermo Scientific), respectively. The extracted DNA samples were stored at −20 °C before downstream analysis. For microbial abundance assessment, triplicate qPCR assays were performed on a CFX96 Real-Time System (Bio-rad) to quantify bacterial and fungal gene copies using the 16S rRNA and ITS genes, respectively. The qPCR assays were conducted using a Real-Time PCR detection system (CFX96 Touch, Bio-Rad). A 2 μL DNA sample was added to 18 μL master mix containing 0.6 μL of each primer (300 nM), 10 μL of iQ™ SYBR® Green Supermix (Bio-Rad), and 6.8 μL of UltraPure™ DNase/RNase-Free distilled water (Invitrogen). For each primer set (338F/518R for bacteria [37], ITS86F/ITS4R for fungi [38]), a linearized plasmid standard (gBlocks, IDT technologies) containing the target region was used to create a standard curve. The thermal profile for bacterial qPCR was as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, and 64 °C for 30 s. The thermal profile for fungal qPCR was as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min. Bacterial qPCR efficiency was 99.8 %, with $R^2 > 0.999$. The fungal qPCR efficiency was 95.3 %, with $R^2 > 0.994$.

DNA samples were normalized to 20 ng/μL for library preparation and sequencing at MrDNA (TX, USA) on a MiSeq platform (Illumina). Libraries for bacteria were constructed using primers 338F (ACTCCTACGGGAGGCAGCAG) [39] and 806R (GGACTACHVGGGTWTCTAAT) [40], and for fungi using primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2R (GCTGCGTTCTTCATCGATGC) [41]. The raw sequence data are available at the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB47068 (https://www.ebi.ac.uk/ena/browser/view/PRJEB71609). Raw sequence data were analysed by QIIME2 (version 2019.10) as described previously [32]. The downstream analyses of bacterial and fungal communities were performed in RStudio (R version 4.0.4) using the phyloseq package [42] and the vegan package [43]. Alpha diversity of bacteria and fungi was assessed on rarefied datasets (at a depth of 70,621 and 66,498 reads per sample, respectively, as shown in Fig. S1) by calculating the Shannon index and observed OTU richness.

2.6. Statistical analysis

Statistical analysis was performed in RStudio (R version 4.0.4). To assess the effects of OA addition on soil physicochemical properties, soil EPS content, and microbial gene abundance, one-way ANOVA (aov() function, alpha = 0.05) was employed, followed by pairwise post-hoc comparisons (TukeyHSD() function, family-wise error rate 5 %). For investigating the effects of types of OA addition, seasons, and years on aggregate formation (GMD) and stability (MWD), a three-way ANOVA was conducted (alpha = 0.05), followed by pairwise post-hoc comparisons (TukeyHSD, family-wise error rate 5 %). Given that GMD and MWD were mainly influenced by the type of OA addition, we did not differentiate among seasons and years in the presentation of the results for GMD and MWD (statistical results are shown in Table S1). Assumptions of normality of residuals and equality of variances were verified for each ANOVA model. To explore EPS patterns and their relationships with soil physicochemical properties, redundancy analysis (RDA) was employed to identify which properties were significantly associated with EPS concentration in the soil (rda() function in vegan package) [43]. Prior to RDA, forward selection was applied to reduce the number of soil physicochemical variables that were inter-correlated. The variance inflation factors (VIFs) of the remaining soil variables in the RDA model were also checked, and VIFs were all lower than 10. Spearman correlations (cor.test() function), which reveal monotonic relationships between two continuous or ordinal variables, were calculated to evaluate potential associations between EPS concentration and soil aggregation (i.e., between EPS and GMD, and between EPS and MWD). For the Spearman correlation analysis, the closest sampling time

point of GMD and MWD to the EPS sampling point was considered, given different sampling time points. Additionally, Spearman correlations were explored between soil microorganisms (at the order level, >1 % relative abundance) and soil EPS. Only robust correlations were considered ($|\rho| \geq 0.6$, $P < 0.05$), based on Barberán et al. [44] who used threshold $|\rho| \geq 0.6$ and $P < 0.01$ to indicate robust Spearman correlation of soil microorganisms. To assess the difference in alpha diversity between treatments, we applied a nonparametric Kruskal-Wallis test (kruskal.test() function), followed by Wilcoxon tests for pairwise comparisons (pairwise.wilcox.test() function). Adjusted (Holm) *P*-values were reported, considering the overall number of comparisons, to control the inflation of Type I errors (false positive results). Principal coordinates analysis (PCoA, ordinate() function in phyloseq package) was used to visualize Bray-Curtis dissimilarities of bacterial and fungal communities (presented in the Supplementary Information Fig. S2) [45]. We analysed order-level taxa to identify differential relative abundances across treatments using a multinomial regression model. This method is specifically employed to handle compositional data with sampling zeros [46] and was implemented using Q2-songbird plugin in QIIME2, following the procedure outlined on GitHub (https://github.com/biocore/songbird). Fitted multinomial models with experimental parameters were compared against null models (intercept only) to explore associations between microbial taxon abundances and OA treatment.

3. Results

3.1. Soil physicochemical properties after three-year of treatment

The addition of OAs significantly affected available nitrogen (AN, the sum of NO_3^-, NO_2^-, and NH_4^+) in the soil (ANOVA, $P < 0.01$, Fig. 2A). Specifically, the Gra + Man treatment exhibited a higher AN concentration compared to the control ($P < 0.05$) and compost amended soil ($P < 0.01$, irrespective of compost application dose). Moreover, the Gra

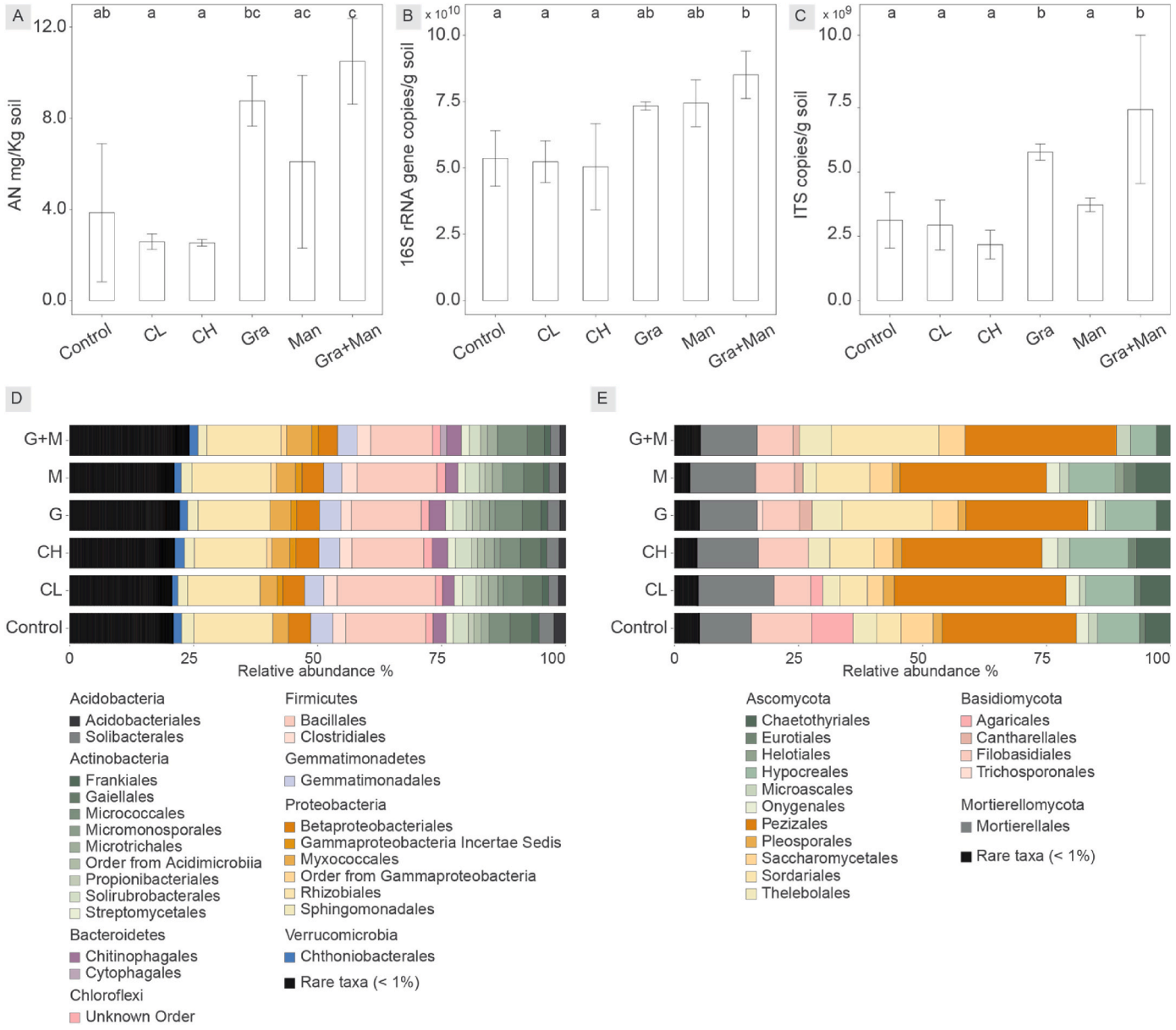

Fig. 2. Available nitrogen (A), qPCR results of bacterial (B) and fungal (C) biomass indicated as 16S rRNA gene copies and ITS gene copies, respectively, and relative abundance of bacteria (D) and fungi (E) at order level after three years of amelioration with different organic amendments (OAs) (mean ± sd; n = 3). Within each panel, boxes with identical letters indicate no significant difference based on the Tukey HSD test.

treatment showed a higher AN concentration than the compost-amended soil ($P < 0.05$, irrespective of compost application dose). However, other physicochemical properties, including pH, EC, WC, water available nutrients, total nutrients, and DOC, did not show significant differences among the treatments. Additional details regarding the values of each physicochemical parameter and statistical results are provided in the supplementary information (Table S2; Table S3).

3.2. Response of microbial abundance and composition to OA application

The abundance of 16S rRNA and ITS gene copies in treatments exhibited a similar trend to that of AN (Fig. 2B and C). Specifically, compost addition did not significantly influence the abundance of soil bacteria ($P < 0.05$) or fungi ($P < 0.05$) compared to the control, while the Gra, Man, and Gra + Man tended to have higher gene copies of bacteria and fungi. Microbial community composition, as indicated by Bray-Curtis dissimilarities, differed among treatments, although alpha diversity did not show significant differences (Fig. S2). The Gra + Man had relatively fewer Acidobacteriales (phylum Acidobacteria) and relatively more Cytophagales (phylum Bacteroidetes) compared to the control soil (Fig. 2D; multinomial regression, Fig. S2D). The relative abundance of the fungi Sordariales (phylum Ascomycota) was significantly higher in the Gra + Man than in other treatments (Fig. 2E; multinomial regression, Fig. S2H). The abundance of Agaricales (phylum Basidiomycota) was significantly lower in all OA-amended soils compared to the control soil. Additionally, the relative abundance of Cantharellales (phylum Basidiomycota) was higher in the Gra, Man, and Gra + Man than in the control. In contrast, the soil amended with compost had similar or lower proportions of Cantharellales than the control.

3.3. Soil EPS, aggregate formation, and aggregate stability after three-year of treatment

Confocal laser scanning microscopy (CLSM) imaging revealed the presence of EPS in the soil. Consistent patterns were observed across all treatments: certain soil aggregates had living microorganisms without detectable polysaccharides (Fig. 3B and C), while others had both living microorganisms and evident polysaccharides (Fig. 3E and F). These observations underscore the variability among soil microorganisms in their ability to accumulate EPS, emphasizing the highly heterogeneous distribution of EPS in the soil. In addition to CLSM images, we quantified EPS concentrations in different treatments. The Gra, Man, and Gra + Man treatments had higher amounts of EPS carbon (EPS-C) and nitrogen (EPS-N) than the control and compost-amended soil (Fig. 4A and B). EPS-C and EPS-N contents were used as proxies for polysaccharides and proteins, respectively. Notably, compost, irrespective of application dose, did not affect the EPS concentration in the soil. The ratio between the concentration of EPS-C and EPS-N remained consistent across all treatments (Fig. 4C).

In general, the addition of OAs significantly increased the percentage of macroaggregates (>2 mm) compared to the unamended soil (Fig. S3). Specifically, the addition of grass and manure, especially in combination, enhanced aggregate stability (Fig. 5A and D) compared to the control, while the addition of compost had no significant effects on MWD and GMD. Strong and positive correlations (Spearman correlation coefficient $\rho > 0.6$) were observed between EPS and MWD (Fig. 5B and C), as well as between EPS and GMD (Fig. 5E and F), suggesting that EPS content played a supportive role in the formation and stability of soil aggregates.

3.4. Correlations between soil physicochemical and microbial characteristics and soil EPS

The RDA was performed on soil physicochemical properties and EPS (EPS-C and EPS-N contents) after forward selection, retaining AN, DOC, TK, TP, and EC in the RDA model (Table S4). Soil EPS content exhibited significant positive correlations with AN ($P < 0.001$) and EC ($P < 0.01$), while a significant negative correlation was observed with DOC ($P < 0.05$). Additionally, EPS content showed significant positive correlations with bacterial (ANOVA, $P < 0.01$) and fungal gene copy numbers (ANOVA, $P < 0.01$). The relative abundance of nine orders (belonging to five phyla) of bacteria and five orders (belonging to two phyla) of fungi were strongly ($|\rho| \geq 0.6$) and significantly ($P < 0.05$) correlated with EPS concentration (Fig. 6). The total relative abundances of the bacterial groups correlated with EPS was less than 0.5 %, while the total relative abundances of fungi correlated with EPS reached 22 %.

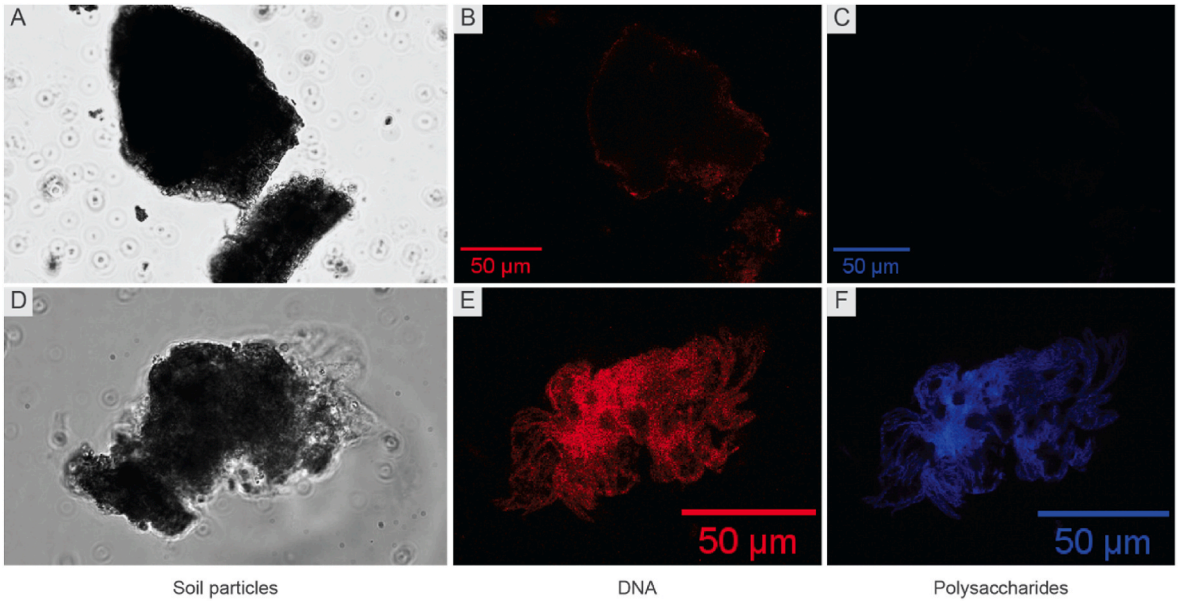

Fig. 3. Confocal laser scanning microscopy (CLSM) of two soil particles (particle one: A, B, C; particle two: D, E, F). DNA (B and E) and polysaccharides (C and F) of the particles were stained and are shown in red and blue colors, respectively.

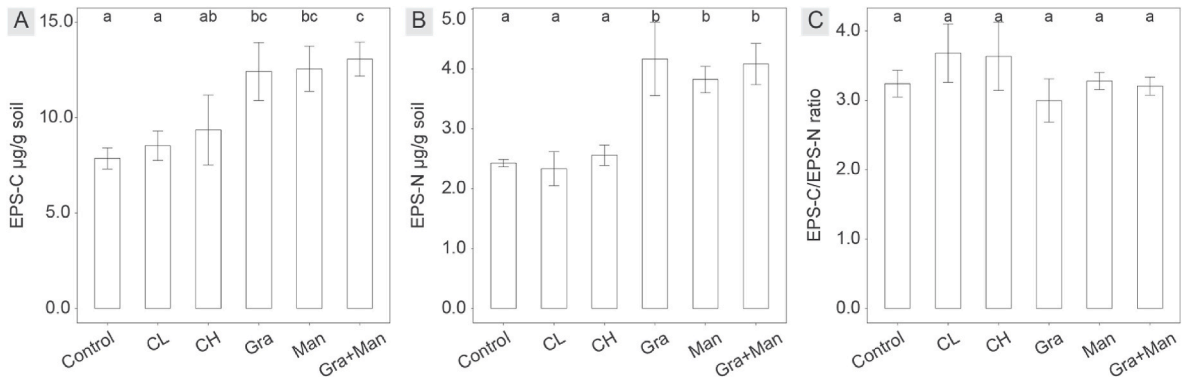

Fig. 4. Polysaccharide concentration (µg/g soil) indicated by EPS-C (A) and protein concentration (µg/g soil) indicated by EPS-N (B), and their ratio (C) after three years of amelioration with different organic amendments (OAs) (mean ± sd; n = 3).

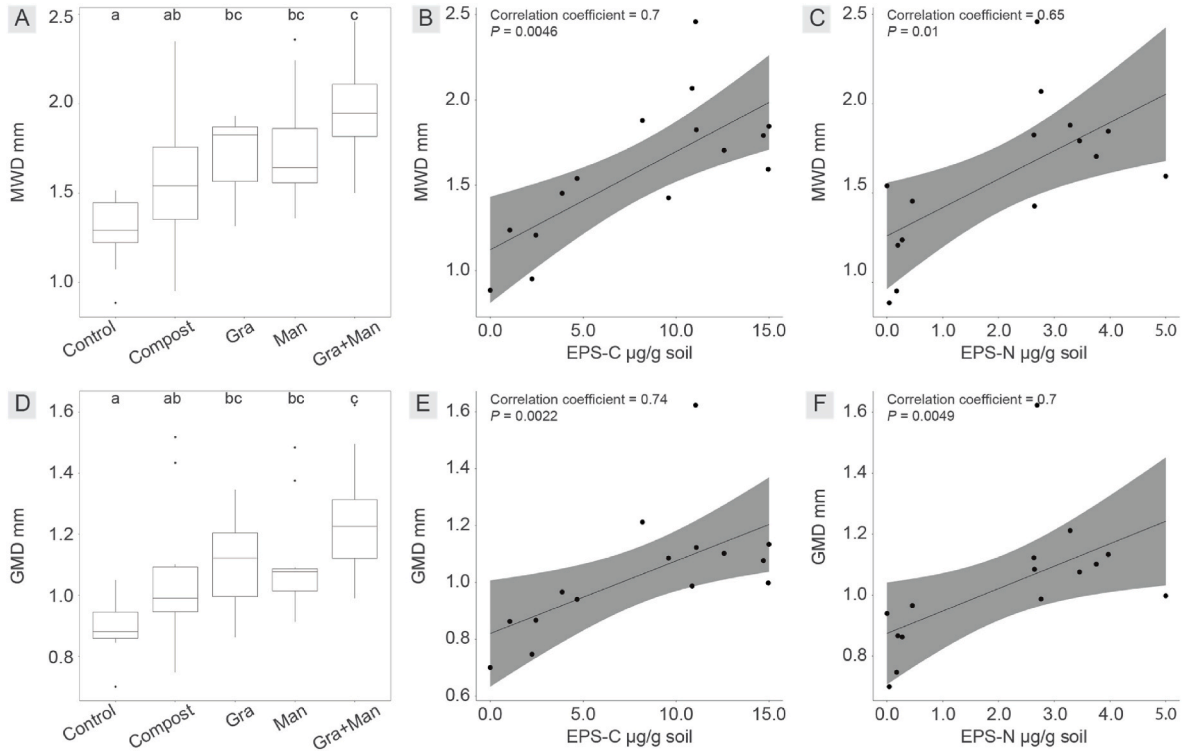

Fig. 5. Aggregate stability expressed as mean weight diameter (MWD, A) and its Spearman correlation with EPS-C (B) and EPS-N (C). Aggregation formation expressed as geometric mean diameter (GMD, D) and its Spearman correlation with EPS-C (E) and EPS-N (F). The box in the boxplot represents the first and the third quartile, and the horizontal line in the boxplots represents the median of the 12 replicates.

4. Discussion

4.1. The addition of grass, manure, and their combination enhance soil EPS and soil aggregation

In this study, we show that soils amended with mown grass (Gra), cattle manure (Man), and the combination of mown grass and cattle manure (Gra + Man) exhibited higher EPS concentrations than the compost-amended and control soils. This aligns with our hypothesis that the availability and accessibility of C substrates regulate microbial EPS production, with labile C substrates exerting a greater impact on EPS production than recalcitrant ones. Compost is an organic material that has undergone biological breakdown into a relatively homogenous and stable form, while grass and manure are fresh organic materials that have not yet degraded. Consequently, compared to grass and manure, compost contained a higher fraction of recalcitrant OM, which included aliphatic and aromatic components with high hydrophobicity [26]. Olagoke et al. [47] observed a similar trend, reporting that the addition of more labile C substrate (i.e., starch) resulted in a higher EPS concentration in the soil compared to the addition of less labile C substrate (i.e., cellulose), especially in soils with high clay content. In such soils, microorganisms may experience slower utilization rates of C substrates due to reduced oxygen availability and limited accessibility of C substrates compared to sandy soils [48,49]. The decomposition of OM and the release of nutrients from compost may play a role in EPS production. Hale et al. [10] suggested that compost might have prolonged effects on EPS production by promoting sustained labile C inputs into the soil, and we believe this merits further investigation. It should be noted that all treatments received a similar total amount of available nutrients, as adjustments were made using mineral fertilizers. We recommend that future experiments include control and OA-amended soils without the addition of mineral fertilizers. This approach would enable us to explore

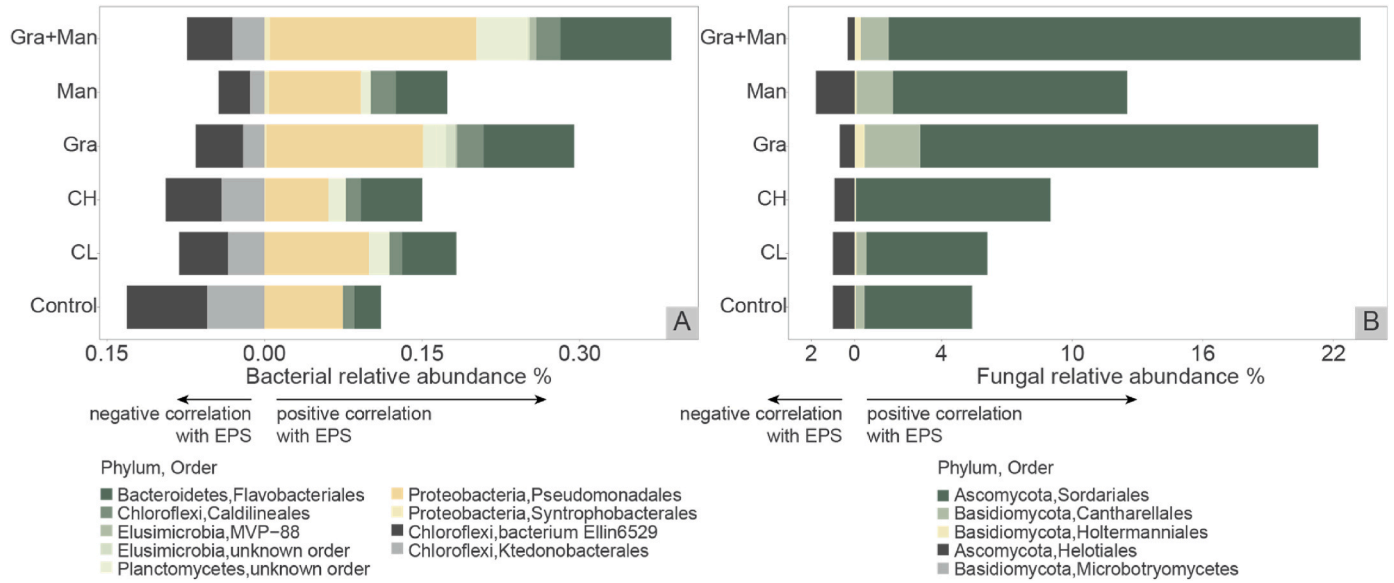

Fig. 6. Total relative abundance of soil bacterial (A) and fungal (B) orders that significantly correlated with soil EPS (sum of EPS-C and EPS-N contents). Included orders showed strong ($|\rho| \geq 0.6$) and significant ($P < 0.05$) correlations with EPS.

the effects of mineral fertilizers and their cross-effects with organic amendments on the soil.

The addition of grass, manure, and their combination increased the stability of soil aggregates. We observed a positive correlation between soil aggregation (as indicated by GMD and MWD) and both EPS-C and EPS-N. This suggests that the contents of EPS-polysaccharides and EPS-proteins potentially contribute to the formation and stability of soil aggregates. In general, EPS-protein content is often considered more crucial than EPS-polysaccharides for aggregate stability [5,21,47]. The presence of hydrophobic R groups in amino acids plays a key role in surface hydrophobicity, providing architectural stability and protecting fragile polysaccharides against disruption from rapid wetting [50,51]. Additionally, these hydrophobic amino acids (such as phenylalanine and tyrosine) contribute to the formation of adhesive peptides, enhancing the cohesive strength of materials produced by soil organisms [52]. However, our findings reveal that EPS-polysaccharides exhibit stronger and more significant correlations with aggregate formation and stability compared to EPS-proteins. We speculate that this could be attributed to the formation of rigid bonds between EPS-polysaccharides and soil ions (e.g., Ca^{2+}) and inorganic C (carbonate binding), altering the molecular structure of EPS and ultimately leading to an increase in soil aggregate stability [10,53,54]. The robust positive correlation between EPS and GMD/MWD underscores the pivotal role of microbial EPS in soil aggregation. Managing soil EPS, such as through the addition of OAs with labile C and fresh plant input, could be a potential strategy for maintaining and improving soil structure [5,9].

4.2. Soil microbial abundance and N availability contribute to soil EPS

In our field experiment, a positive correlation was observed between microbial abundance (indicated by gene copy numbers) and EPS concentration. Wu et al. [55] observed enhanced EPS production during the microbial growth phase in a batch experiment. During the growth phase, the majority of sugar substrates are phosphorylated into sugar-6-phosphates and degraded through glycolysis by microorganisms. Some of these sugar-6-phosphates can be converted into sugar-1-phosphates by phosphoglucomutases [19]. These sugar-1-phosphates serve as central metabolites for forming sugar nucleotides (such as UDP-glucose, UDP-galactose, and dTDP-rhamnose), from which the majority of EPS is synthesized [19,56,57]. This mechanism may explain the observed positive correlation between microbial abundance and soil EPS in our data.

Negative correlations between EPS production and microbial abundance have also been reported, especially under drought conditions or when temperature and salt stress are applied [15]. These observations do not necessarily contradict our findings. The allocation of carbon and energy resources to microbial growth or EPS formation is a survival strategy for microorganisms. When exposed to environmental stresses, microorganisms may allocate more carbon to EPS production than to growth since EPS advantageously enables the storage of carbon, nutrients, and energy [3]. However, when the growth of microorganisms is not threatened by environmental stresses, as was the case in the current field study, microbial growth and EPS production are not “competing” processes, and EPS is coupled with microbial growth but probably with lower EPS production efficiency (i.e., C allocated to EPS synthesis vs. C allocated to microbial cell growth) than would be achieved under environmental stress.

We expected that there would be more EPS in soils with more available nutrients. We indeed found that for AN, there was a significant positive correlation with soil EPS. N management has been widely used to regulate EPS production in different research fields (e.g., water research and molecular microbiology for biosynthesis), but contradicting effects have been reported: a range of low to high N inputs have all been demonstrated to promote microbial EPS production [55,56,58–61]. Therefore, it remains difficult to generalize the dependence of soil EPS on N availability. Wu et al. [55] and More et al. [62] reported that microbial EPS production was positively related to microbial growth and N supply. It is important to note that an increase in N in the soil that causes enhanced microbial growth does not necessarily result in higher EPS production. Microbial growth depends on the C:N ratio, and an excess of available N can result in the rapid mineralization of OM [63]. This can lead to EPS degradation since EPS can serve as C sources. Furthermore, N starvation can also promote EPS production under the condition that microbial growth is ensured. Under N limitation or a high C:N ratio, microorganisms may allocate more C to EPS production than cellular growth. This is because the availability of N is insufficient for protein synthesis, and the excess energy from a surplus of C sources also supports EPS production, particularly polysaccharide production [64,65]. Ajao and co-workers [59] observed an increase in microbial EPS production by applying N limitation. Still, they observed a decrease in microbial EPS production in an environment unsuitable for microbial growth such as a C:N ratio of 100. Investigating the role of N and the

effects of C:N ratio through adding OAs and fresh plant inputs differing in C:N ratios on EPS production in the soil and its potential application for managing EPS and soil structure merits further investigation.

4.3. Specific bacterial and fungal taxa that are correlated with EPS concentration in the soil

We identified several bacterial taxa whose relative abundance positively correlated with EPS. Among these identified taxa, Pseudomonadales and Elusimicrobia were particularly related to EPS concentration in the soil. Pseudomonadales are well-recognized and commercially available EPS producers [66–68]. In soil, the genus *Pseudomonas* can produce biofilms [69]. Pseudomonadales can encode proteins involved in the metabolism of cyclic dinucleotide (c-di-GMP), which plays a vital role in the regulation of EPS production [70]. Two orders from the Elusimicrobia phylum also positively correlated with EPS. Elusimicrobia is an enigmatic and recently described bacterial phylum [71]. Elusimicrobia can fix N [72] and are capable of synthesizing common energy-storage polysaccharides, with several genes encoding enzymes for starch or glycogen metabolism [71]. This indicates that Elusimicrobia may be an EPS producer or indirectly involved in the EPS matrix through nutrient (particularly N) interactions with other microorganisms.

Microorganisms that correlate with soil EPS are not necessarily EPS producers. In some cases, EPS produced by certain bacteria can serve as resources or act as growth substrates for other bacteria in the soil [73]. For instance, Planctomycetes, Chloroflexi, and Flavobacteriales are potential EPS degraders, and they showed positively or negatively correlations with soil EPS in our study. Planctomycetes are considered K-selected bacteria with efficient cell metabolism and strong competitive ability, growing slowly on recalcitrant complex substances [74]. They are recognized as primary degraders of complex heteropolysaccharides in the soil [73]. Chloroflexi, particularly the Ktedonobacterales, play a role as heterotrophic oligotrophs in soils. They usually contain numerous exoenzymes, such as chitinase, glucuronidase, galactosidase [75], and proteases [76]. This suggests that Chloroflexi primarily grow on complex polysaccharides and proteins [77–79]. Flavobacteriales constitute a bacterial group often associated with the capacity to degrade complex organic compounds or macromolecules in the soil [80]. Flavobacterium strains can produce glucosamine-6-phosphate deaminases [80], catalysing the reversible isomerization and deamination of D-glucosamine 6-phosphate (important metabolites for EPS synthesis [57]) into D-fructose 6-phosphate [81]. Therefore, Flavobacteriales may either consume EPS or suppress EPS production.

Five fungal orders were identified whose relative abundance correlated with EPS concentration: Sordariales, Cantharellales, Holtermanniales, Microbotryomycetes, and Helotiales, belonging to two fungal phyla, Ascomycota and Basidiomycota. Both Ascomycota and Basidiomycota are considered saprotrophic fungi. While these fungi have been shown to produce EPS in other studies (e.g., Rashid et al. [82]) and can utilize bacterial EPS as C sources [83], the specific roles of the fungal orders identified in this study in the soil EPS matrix have been rarely documented. These fungi may be directly or indirectly involved in building the soil EPS matrix, especially in interactions with EPS-producing/degrading bacteria. It has been reported that bacteria can form biofilms around fungal hyphae by producing EPS and altering EPS composition to facilitate subsequent adherence [84].

We emphasize that correlations between soil microorganisms and EPS concentrations do not reveal the causal mechanisms governing EPS dynamics in the soil. For future studies, we recommend the utilization of other statistical methods, such as structural equation modeling [85], designed to reveal causal connections. Additionally, experimental validations of these correlations are crucial to uncover the role of soil microorganisms in regulating EPS production/degradation and soil aggregation. However, due to the inherent complexity of the soil system, understanding the functioning of certain microbial groups and their ecological relevance remains significantly challenging. To address this, we recommend employing a synthetic community (SynCom) representing core soil microbiomes, with the addition or elimination of one group [86,87], to enable a step-wise investigation of their role in regulating EPS content in the soil and soil aggregation.

5. Conclusion

In this field experiment, the addition of grass, manure, and their combination increased soil EPS concentration compared to the unamended soil, while the addition of compost, regardless of the application dose, had the least impact on soil EPS concentration compared to the unamended soil. Grass and manure, especially when combined, also improved soil aggregation and stability. Soil EPS showed a positive correlation with AN and microbial biomass. The total relative abundance of bacteria and fungi that positively correlated with EPS was higher in the soil amended with grass, manure, and their combination than the unamended and compost-amended soils. The correlation observed between soil microbes and soil EPS in this study warrants further experimental validation to elucidate the role of soil microorganisms in building the EPS matrix under various environmental conditions, such as different C sources, nutrient limitations, and extreme climates. Future research focused on unravelling the interactions between bacteria and fungi in the EPS production process is also recommended. Our work demonstrates how the addition of various OAs, such as grass, cattle manure, or their combination, can enhance soil EPS production and, consequently, improve soil aggregate stability on a field scale. These findings offer valuable insights for designing and implementing sustainable agricultural management practices, particularly through the reuse of organic residues to regulate EPS production. In the face of climate extremes, such as heatwaves and periodic droughts, our results become even more significant. They highlight the importance of enhancing soil structure, as this leads to stable soil aggregates and increased resistance to environmental stress.

CRediT authorship contribution statement

Yujia Luo: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Juan Bautista Gonzalez Lopez:** Formal analysis, Data curation. **H. Pieter J. van Veelen:** Writing – review & editing, Methodology. **Dirk-Jan Daniel Kok:** Writing – review & editing, Formal analysis. **Romke Postma:** Writing – review & editing, Resources. **Dirk Thijssen:** Resources. **Valentina Sechi:** Writing – review & editing, Supervision, Conceptualization. **Annemiek ter Heijne:** Writing – review & editing, Supervision, Conceptualization. **T. Martijn Bezemer:** Writing – review & editing, Supervision, Conceptualization. **Cees J.N. Buisman:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejsobi.2024.103624.

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