Dynamics of active potential bacterial and fungal interactions in the assimilation of acidobacterial EPS in soil

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ABSTRACT

Acidobacteria are one of the most abundant and ubiquitous bacterial phyla in soil, but the mechanisms underpinning their ecological success remain unknown. *Acidobacteria* produce copious amounts of extracellular polymeric substances (EPS) with unique sugar composition that can be used as a nutrient source for other microorganisms. Here, we investigated the assembly and potential interactions of the active bacterial and fungal communities in soil treated with the EPS of the *Acidobacteria Granulicella* sp. strain WH15 (WH15EPS) as a carbon source by the stable isotope probing (SIP) approach during a 35-day period. WH15EPS was mainly assimilated by *Planctomycetes*, *Verrucomicrobia*, *Ascomycota* and *Basidiomycota* and analysis suggested overall relationships between the kingdoms. Our study revealed active potential interactions between microorganisms in their natural habitat. In addition, the structure of the co-occurrence network of active microorganisms able to metabolize WH15EPS differed from those of the control treatments, demonstrating that hidden potential interactions can be unraveled by more specific and targeted metabolism studies.

1. Introduction

Acidobacteria are one of the most abundant bacterial phyla in soil, constituting 20–50% of the soil bacterial community (Kuramae et al., 2012; Navarrete et al., 2013; Pan et al., 2014; Kielak et al., 2016a, 2016b). However, little information is available on the physiology, ecological function, and impact of *Acidobacteria* on the soil environment (Kielak et al., 2016a) because their slow growth under standard laboratory conditions has resulted in a relatively small number of cultured representatives. Consequently, the factor(s) responsible for the prevalence and successful adaptation of *Acidobacteria* and their relationships with other soil-inhabiting microbes remain unknown (Kielak et al., 2016a).

Network analysis studies using culture-independent sequence data have shown that *Acidobacteria* species co-occur with other *Acidobacteria*, *Proteobacteria*, *Actinobacteria* and *Planctomycetes* (Lupatini et al., 2014; Navarrete et al., 2015). However, no study has investigated the active potential interactions of *Acidobacteria* with other bacteria and/or fungi in co-occurrence networks associated to the use of an acidobacterial biopolymer.

Acidobacteria such as strains Ellin6076 and Ellin345 (Ward et al.,

2009), *Terriglobus tenax* (Whang et al., 2014) and *Granulicella* sp. strains WH15 and 5B5 (Kielak et al., 2017) produce extracellular polymeric substances (EPS). EPS are biopolymers secreted by a variety of microorganisms and mainly comprise carbohydrates, proteins and DNA. EPS play an important role in numerous bacterial physiological traits and ecological adaptations, especially in bacteria living in communities and with complex interactions with other organisms. The role of EPS depends on the ecological niche and natural dwelling environment of the microorganism (Costa et al., 2018). EPS may positively impact bacterial survival and function by enhancing bacterial quorum sensing, thus stimulating important metabolic activities and consequently promoting syntrophic interactions (Wingender et al., 1999). EPS production has been implicated in the long-term survival of *Acidobacteria* in several environments due to its protective properties (Kielak et al., 2017). Among *Acidobacteria*, EPS have only been isolated and chemically characterized from *Granulicella* sp. strains WH15 and 5B5, and the emulsion capacity of these EPS suggests potential industrial and environmental bioremediation applications (Kielak et al., 2017). EPS also serve as nutrient sources for other microorganisms (Flemming and Wingender, 2010), and the use of EPS from microbes other than *Acidobacteria* as a nutrient has been investigated in cross-feeding

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experiments.

The assimilation of EPS as a carbon source by active microorganisms can be investigated with stable isotope probing (SIP). SIP is a powerful technique that evaluates the incorporation of compounds labeled with heavy isotopes, such as ^{13}C , ^{18}O and ^{15}N , into the cell components of microorganisms metabolizing a specific substrate. Thus, SIP reflects the active microorganisms involved in the metabolism of a specific compound. SIP has been used to investigate the microorganisms responsible for the degradation of different compounds in many environments (Madsen, 2006), including labeled glucose (Verastegui et al., 2014), methanol (Ginige et al., 2004), phenol (Padmanabhan et al., 2003), salicylate and anthracene (Singleton et al., 2005). The use of SIP to investigate EPS has been limited to a single study identifying microorganisms that assimilate the EPS of *Beijerinckia indica* (Wang et al., 2015). In this context, in the present study, EPS from *Granulicella* sp. strain WH15 was applied as a carbon source to soil sampled from the site where this strain was isolated, and the active bacterial and fungal community assemblages and potential interactions in the soil during a 35-day period were evaluated by SIP. We hypothesized that only a selection of specific microbes with a wide range of glycoside hydrolases would be able to incorporate WH15EPS.

2. Material and methods

2.1. Soil sampling

Four organic topsoil samples were collected in the spring of 2017 from the Wolfheze forest in the Netherlands. Samples were taken from topsoil (0–5 cm) adjacent to fallen tree trunks. Wolfheze forest is a mixed forest site (managed by Natuurmonumenten) close to Doorwerth village, the Netherlands (N 51° 58' 60", E 15° 47' 60", altitude 49 m). The forest has developed on glacial sandy deposits with oak (*Quercus robur* L.), beech (*Fagus sylvatica* L.), birch (*Betula* sp.) and pine (*Pinus sylvestris* L.) as major species (Valášková et al., 2009). In order to collect samples, we removed the superficial litter layer and collected the hummus and fermentation layers of the soil, rich in organic matter, with a shovel. The sampling was performed in regions close to tree trunks, since *Granulicella* strains can be also isolated from fallen oak trunks (Valášková et al., 2009).

The collected samples were pooled, sieved (4 mm mesh) and immediately used for SIP incubation with EPS from *Granulicella* sp. strain WH15 (WH15EPS). The physicochemical properties of the organic topsoil samples were determined by Eurofins Agro BV, Wageningen, NL. The organic topsoil employed in the experiment presented pH 3.1 ± 0.1 , $55 \pm 3.5\%$ of organic matter, $16.5 \pm 3.2 \text{ g kg}^{-1}$ of total N, $352 \pm 4.2 \text{ mg kg}^{-1}$ of available N, cation exchange capacity (CEC) of $21.4 \pm 4.2 \text{ cmol kg}^{-1}$, $42.7 \pm 6.4 \text{ mg kg}^{-1}$ of available P, C/N ratio of 20 ± 5.7 . Maximum water holding capacity (WHC) of sieved organic topsoil was $1.85 \pm 0.03 \text{ g g}^{-1}$ dry soil. Organic matter, Total N and CEC were measured by Near-infrared (NIR) reflectance spectroscopy (triple-short calibration); available P was extracted with acid ammonium oxalate solution (pH 3) and measured by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES); pH was measured by potentiometry- CaCl_2 . Maximum WHC was measured by soaking 25 g of the organic topsoil soil ($n=8$) in 25 ml deionized water and then draining for 5 h. Next, organic topsoil samples were dried at 105°C for 24 h. WHC was determined by the difference between the mass of the oven-dried and the wet topsoil samples.

2.2. [^{13}C]-labeled and unlabeled EPS production

Granulicella sp. strain WH15 was grown on PSY5 solid medium (Campanharo et al., 2016) containing 3% (wt/vol) fully ^{13}C -labeled glucose as the sole carbon source or unlabeled glucose for unlabeled control EPS production. The plates were incubated at 30°C for 3 days and then at 20°C for 27 days. The polysaccharide portion of EPS was

extracted and purified according to the formaldehyde–NaOH method described by Liu and Fang (2002), with modifications. The method was chosen due to the fixing properties of formaldehyde, which prevents cell lysis. EPS and cells of strain WH15 (~5 ml) were scraped from the plates into 50-ml Falcon tubes, and the volume was adjusted to 10 ml with sterile water. Sixty microliters of 36.5% formaldehyde was added to each sample and incubated at 4°C for 1 h. Next, 4 ml of 1 M NaOH was added and incubated at 4°C for 3 h. The cells were then pelleted by centrifugation at $9000 \times g$ for 40 min. The supernatants were filtered (0.2 μm membranes, Millipore) at room temperature, and mono-saccharides and other low molecular weight metabolites were removed by dialysis in SnakeSkin™ Dialysis Tubing (3500 Da) (Thermo Fisher Scientific, Massachusetts, USA) against demineralized water at 4°C for 48 h. The solutions were frozen at -80°C for 12 h and then freeze-dried at -80°C for 72 h. Before freeze-drying, the DNA concentration in the EPS solution was determined in a Qubit fluorometer using a broad-range Quant-iT™ dsDNA Assay Kit (Invitrogen, Carlsbad, California, USA). EPS protein concentrations were determined by a Pierce™ Modified Lowry Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). The total carbohydrate content was estimated by the phenol-sulfuric acid method (DuBois et al., 1956) modified for 96-well plates (Masuko et al., 2005) with glucose as the standard. After purification, the EPS contained ~99% carbohydrates, ~1% protein and undetectable amounts of DNA. The monosaccharide composition of WH15EPS was previously characterized (Kielak et al., 2017) and comprises xylose (41.8%), mannose (10.25%), glucose (13.55%), galactose (26.12%), rhamnose (0.065%), glucuronic acid (8.09) and galacturonic acid (0.085%).

2.3. Stable isotope probing (SIP) incubation

One milliliter of Milli-Q sterile water was added to the freeze-dried EPS immediately before inoculation in soil to facilitate a homogeneous distribution within the soil. Five grams (wet weight) of soil with 0.05% (wt/wt) WH15EPS (labeled and unlabeled controls) or without EPS were added to a 120-ml bottle, which was sealed with a butyl rubber stopper and incubated at room temperature (22°C) in the dark at 70% water-holding capacity (WHC). Each treatment (labeled EPS, unlabeled EPS and control without EPS) had six replicates. All vials were uncapped and aired every 4 days to maintain oxic conditions and prevent $^{13}\text{CO}_2$ cross-feeding. The vial headspace CO_2 was monitored daily via gas chromatography (GC) (Trace GC Ultra, Thermo Fisher Scientific, Massachusetts, USA). For incubations with [^{13}C]-labeled EPS, the headspace CO_2 $^{13}\text{C}/^{12}\text{C}$ ratio was monitored via GC combustion isotope ratio mass spectrometry (GC/C/IRMS) (GC IsoLink II™ IRMS System, Thermo Fisher Scientific, Massachusetts, USA). For DNA extraction, 0.5 g was sampled from the vials on days 8, 24 and 35, which corresponded to 10, 25 and 43% $^{13}\text{CO}_2$ headspace enrichment, respectively. The differences in CO_2 emissions on the different days were analyzed by analysis of variance (ANOVA one-way repeated measurements) using mixed-effects models ('lmerTest' (Kuznetsova et al., 2017) and 'psycho' (Makowski, 2018) packages in R).

2.4. DNA extraction and fractionation

DNA was extracted from 250 mg of soil with or without ^{13}C -labeled/unlabeled substrates with the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc) according to the manufacturer's instructions. DNA was quantified by a spectrophotometer (NanoDrop™ 2000, Thermo Fisher Scientific, Massachusetts, USA) and visualized by 1.0% agarose gel electrophoresis and ethidium bromide staining. For gradient fractionation, 2 μg of DNA was combined with CsCl (1.72 g ml^{-1}) and gradient buffer (100 mM Tris-HCl pH 8.0, 100 mM KCl, 1 mM EDTA) in an ultracentrifugation tube (PA UltraCrimp 1.8 ml, ThermoFisher Scientific, Massachusetts, USA) (Neufeld et al., 2007) and ultracentrifuged at $125,395 \times g$ (Discovery 120SE ultracentrifuge, ThermoFisher

Scientific, Massachusetts, USA) under vacuum at 20 °C for 65 h. Gradient fractionation resulted in 18 DNA fractions of approximately 100 µl each. The density of each fraction was measured with a refractometer (AR200, Reichert Technologies, New York, USA) to confirm gradient formation. DNA was precipitated from the CsCl with polyethylene glycol solution (30% PEG6000, 1.6 M NaCl) and glycogen (20 µg/µl), washed with 70% ethanol, and eluted in 30 µl of 10 mM Tris-HCl buffer, pH 8.0. The DNA concentration of each fraction was determined in a Qubit 4 Fluorometer (ThermoFisher Scientific, Massachusetts, USA) using a Quant-iT™ dsDNA HS Assay Kit (Invitrogen, Carlsbad, California, USA). The unlabeled substrate incubations were used as controls to determine the expected position of labeled soil DNA in the CsCl density gradients. “Heavy” fractions were chosen at densities at which little or no total DNA was detected in the control sample fractions.

2.5. PCR, sequencing and sequence processing of 16S rRNA gene and ITS data

Amplicon library preparation and high-throughput sequencing were performed using the “heavy” DNA fractions from samples belonging to the labeled EPS treatment pooled within each sample replicate as well as the total DNA of both the amended and unamended controls. The V3-V4 region of the 16S rRNA gene and the internal transcribed spacer 1 (ITS1) region were targeted for bacteria and fungi, respectively. For bacteria, the V3-V4 region of 16S rRNA gene was targeted by using 515F (5'-GTGCCAGCMGCCGCGGTAA-3') as forward primer and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Bergmann et al., 2011) as reverse primer. For fungi, the Internal Transcribed Spacer 1 (ITS1) region was targeted by using primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTCTTCATCGATGC-3') (White et al., 1990). The amplification steps and Illumina MiSeq PE250 sequencing were performed at McGill University and Génome Québec Innovation Centre (Montréal, Québec, Canada). The sequences were deposited in the European Nucleotide Archive (ENA; <https://www.ebi.ac.uk/ena>) under the accession number PRJEB29719.

2.6. Processing and statistical analyses of 16S rRNA gene and ITS data

Raw sequencing data were processed through Hydra pipeline version 1.3.3 (Hollander, 2017) implemented in Snakemake (Koster and Rahmann, 2012). Reads were first quality filtered, by trimming adapters sequences and removing PhiX contaminants, using BBDuk2 from the BBDuk tool suite (Bushnell, 2015). Paired-ends were merged using the fastq_mergepairs option from vsearch (Rognes et al., 2015). The ITS1 region was extracted from ITS sequence dataset using ITSx version 1.011 (Bengtsson-Palme et al., 2013). Next, vsearch was employed to cluster all reads into OTUs through the UPPARSE strategy by dereplication, sorting by abundance with at least two sequences and clustering using the UCLUST smallmem algorithm (Edgar, 2010). Chimeric sequences were detected and removed using the UCHIME algorithm in de-novo mode (Edgar et al., 2011) implemented in VSEARCH. Before dereplication step, all reads were mapped to OTUs with the usearch_global method implemented in VSEARCH to generate an OTU table and converted to BIOM-Format (McDonald et al., 2012). For 16S rRNA sequences, the taxonomic information for each OTU was then added to the BIOM file by aligning the sequences to the SILVA database (release 128) (McDonald et al., 2012) using SINA classifier (Pruesse et al., 2012). For ITS sequences, the taxonomic information was added to the BIOM file by running the RDP Classifier re-trained on the UNITE database release 7.2 (Kõljalg et al., 2013). Fungal OTUs were assigned to functional guilds using the annotation tool FUNGuild (Nguyen et al., 2016). For assignment at the OTU, genus and phylum levels, 97%, 95% and 75% sequence similarity were used as thresholds, respectively (Webster et al., 2009).

2.7. Multivariate analyses of 16S rRNA gene and ITS data

Statistical analyses were performed in RStudio version 1.1.423 running R version 3.4.4 (R Core Team, 2015). OTUs with less than 2 counts across all samples and chloroplast and mitochondrial sequences were discarded. Prior to alpha and beta diversity analyses, the data were rarefied to the size of the smallest sample (4243 reads for the 16S rRNA gene and 7797 for ITS region data). The ‘Phyloseq’ package (McMurdie and Holmes, 2013) was used to calculate the number of observed OTUs, Shannon and Inverse Simpson diversity indices, and Chao1 and ACE diversity estimators. Significant differences in the estimators between treatments were evaluated through parametric and non-parametric tests, including ANOVA, Kruskal-Wallis and Tukey’s HSD tests (package ‘agricolae’ (Mendiburu, 2017)). Bray-Curtis distance matrices constructed using the rarefied datasets were used for principal coordinate analysis (PCoA) using the *capscale* function from the ‘vegan’ package (v. 2.4.6 (Oksanen et al., 2018)). Group dissimilarities were tested by permutational multivariate analysis of variance (PERMANOVA) using the function *Adonis* from the ‘vegan’ package. To compare the structures of the bacterial and fungal communities within treatments, co-inertia (COIA) analysis of the Hellinger transformed datasets (Legendre and Gallagher, 2001) was performed using the function *coinertia* of the package ‘ade4’ (Dray and Dufour, 2007) as described by Schlemper et al. (2017). For this analysis, OTUs with less than 15 counts were excluded, and then grouped at genus level. RV. test function was applied to run Monte-Carlo test for COIA, with 999 permutations. As a result, COIA plots display arrows that illustrate the strength of the relationships between bacterial and fungal communities, which is inversely proportional to length of the arrows. In addition, arrows pointing to the same direction suggest associations between treatments. Potential interactions between bacterial and fungal genera within treatments were investigated via SparCC correlation coefficients, with default parameters and 100 bootstraps (Friedman and Alm, 2012). Networks were built with significant correlations ($P < 0.05$) at $|r| > 0.75$, and co-occurrence was visualized by Cytoscape (Shannon, 2003) and Gephi version 0.9.2 (<https://gephi.org/>). Topological parameters (numbers of nodes and edges and node degree number) were calculated using the network analyzer tool (Doncheva et al., 2012) pre-installed in Cytoscape. Other plots were generated using ‘ggplot2’ (Wickham, 2016) and the *plot* function in R.

3. Results

3.1. CO₂ measurements

The CO₂ emissions of the microcosms were 7500 ppm CO₂/day initially and progressively decreased during incubation. Average CO₂ emissions were 1.3 times higher in the EPS-amended samples than in the unamended controls. At the end of the incubation, the EPS-amended microcosms CO₂ emissions were 1.9 times higher than that of unamended controls. Headspace ¹³C₂O₂ enrichment increased throughout the incubation period, reaching 43% of the headspace in the labeled samples at day 35 (Fig. 1). The differences in CO₂ emissions between treatments and P-values are shown in Table 1.

3.2. Gradient fractionation

The density profiles of the DNA extracted from soils incubated with ¹³C-EPS exhibited small changes compared with those of the control DNA from the ¹²C-EPS incubations (Fig. S1). Among the 6 replicates, two from day 8 (L2D8 and L6D8) had “heavy” DNA in higher-density fractions, probably due to variations in the CsCl density centrifugation.

3.3. Sequencing and alpha diversity

High-throughput sequencing generated 1,250,548 and 828,045

Fig. 1. CO₂ emission. CO₂ production during total incubation period. No EPS: control without EPS; Unlabeled EPS: control containing ¹²C-EPS; Labeled EPS: incubation with ¹³C-EPS; Labeled CO₂ percentage: ¹³CO₂ emitted during ¹³C-EPS sample incubation; water: days when samples were hydrated; air: days when samples were aired. Error bars represent standard error of the mean (n = 6).

Table 1

Contrast between CO₂ emission curves and statistical P-values.

Contrast	Difference	P-value
Unlabeled EPS -Control	1094.9	0.01
Labeled EPS-Control	1058.1	0.02
Unlabeled EPS – Labeled EPS	−36.8	0.99

good-quality sequences for the bacterial 16S rRNA gene and fungal ITS region, respectively. Good's coverage (Table 2) indicated that the number of sequence reads covered 96.2%–98.4% of the bacterial diversity and 99.1%–99.9% of the fungal diversity in the samples at the OTU level (3% dissimilarity). Overall, for the 16S rRNA samples, the “heavy” fraction samples at all time points had significantly lower richness and diversity values than the unamended and ¹²C-EPS-amended controls. Moreover, the ¹²C-EPS-amended samples had lower richness and diversity values than the unamended samples (Fig. S2).

Similar to the 16S rRNA gene samples, the richness and diversity values of the ITS region were lower in the “heavy” fraction samples compared with the controls. This tendency was most obvious at days 24 and 35, whereas at day 8, the richness of the “heavy” fraction was not

Table 2

Good's coverage for 16S rRNA gene and ITS region sequences within treatments.

16S rRNA gene sequences			ITS region sequences		
Treatment	Day	Good's coverage	Treatment	Day	Good's coverage
Control	8	96.2	Control	8	99.3
Unlabeled EPS	8	96.4	Unlabeled EPS	8	99.2
“Heavy” fraction	8	97.4	“Heavy” fraction	8	99.6
Control	24	96.3	Control	24	99.2
Unlabeled EPS	24	96.9	Unlabeled EPS	24	99.3
“Heavy” fraction	24	98.4	“Heavy” fraction	24	99.9
Control	35	96.3	Control	35	99.0
Unlabeled EPS	35	97.0	Unlabeled EPS	35	99.4
“Heavy” fraction	35	98.3	“Heavy” fraction	35	99.8

significantly different from that of the controls (Fig. S3).

3.4. Community structures

PERMANOVA showed that the bacterial communities differed significantly ($P = 1.00 \times 10^{-4}$) between the different treatments, days and treatment:day interactions, with the “heavy” fractions on days 24 and 35 clustering separately from the controls and the day 8 “heavy” fraction in the PCoA plot (Fig. 2a). The bacterial communities clearly clustered according to treatments and days, with the first two axes of the PCoA explaining 70.5% of the variation.

The fungal community patterns at different time points were less clear than those of the bacterial community. The fungal communities were more spread throughout the PCoA plot (Fig. 2b). However, the heavy fractions on days 24 and 35 also clustered separately from the control samples and the day 8 “heavy” fraction. PERMANOVA indicated significant differences (P value = 9.999×10^{-5}) between treatments, days and treatment:day interactions, with the first two axes of the PCoA explaining 27.8% of the variation among samples.

Co-inertia analysis revealed significant co-structures between the bacterial and fungal communities for all treatments ($P < 0.5$). The first 2 co-inertia axes explained 80.4% (“heavy” fraction), 61.8% (EPS amended control) and 59.9% (unamended control) (cumulative projected inertia) of the total variance in the bacterial-fungal assessments (Fig. 2cde). In addition, the bacterial and fungal groups at the genus level that contributed most to the co-variance between samples were identified, which reflected the differences in abundances among time points (Fig. S4).

3.5. Bacterial community beta diversity

Twenty-two groups at the phylum level were observed in all samples. At day 8, the most abundant phylum in all samples was *Proteobacteria* (32.01%–39.7%) (Fig. S5a). At day 24, the separation between microbial communities observed in the PCoA analysis became evident. The most abundant phylum was *Planctomycetes* in the “heavy” fraction (59.79%) but *Proteobacteria* in both control treatments (28.56%–31.23%) (Fig. S5a). At day 35, *Planctomycetes* (60.79%) was still the

Fig. 2. Co-inertia (COIA) and Principal Coordinate Analysis (PCoA) of microbial communities. Co-inertia (COIA) analysis between PCoAs of a) bacterial and b) fungal community composition of “heavy” fractions of samples amended with labeled EPS c) (red), unlabeled EPS d) (blue) and unamended samples e) (green). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

most abundant phylum in the “heavy” fraction samples (Fig. S5a), and *Proteobacteria* (25.65%–25.74%) remained the most abundant phylum in the controls.

At the genus level, 220 groups were found in all samples. At day 8, unclassified *Pedospheareaceae* was the most abundant group in the

“heavy” fraction and the unamended control (12.34% and 13.04%, respectively). In the ^{12}C -EPS-amended treatment, the most abundant genus was *Rhodanobacter* (12.12%) (Fig. 3a).

At day 24, *Singulisphaera* was the most abundant genus in the “heavy” fraction samples (27.79%) but represented only approximately

Fig. 3. Relative abundance of microbial genera. Relative abundance of a) bacterial and b) fungal groups at genus level in “heavy” fraction, EPS amended (Unlabeled EPS) and unamended (No EPS) controls at all time points (days 8, 24, 35). Only genera with >3% abundance are shown.

1.07–1.28% of the sequences in the control treatments. In both control treatments, unclassified *Pedospaeraceae* (10.91%–14.36%) was the predominant group (Fig. 3a).

At day 35, uncultured *Pirellulaceae* (36.21%) was the most abundant group in the “heavy” fraction, followed by *Singulisphaera* (16.22%). In the unamended control, the most abundant group was unclassified *Pedospaeraceae* (16.52%), while in the amended control, the predominant group was uncultured *Pirellulaceae* (12.14%) (Fig. 3a).

3.6. Fungal community beta diversity

At the phylum level, 11 groups were observed in all samples. At days 8 and 24, *Basidiomycota* was the most abundant phylum in all samples (35.92%–43.16%) (Fig. S5b). At day 35, the predominant group was *Basidiomycota* in the “heavy” fraction samples (38.7%) but *Mortierellomycota* in the unamended control (33.36%). In the ¹²C-EPS amended control samples, *Ascomycota* (33.87%) was the most abundant phylum (Fig. S5b).

At the genus level, 282 groups were observed in all samples. At day 8, the most abundant genus was *Scleroderma* in the “heavy” fractions (18.02%) but *Mortierella* in both control treatments (22.39%) (Fig. 3b). At day 24, *Trichoderma* (15.67%) was the most abundant genus in the “heavy” fraction samples. In both control treatments, *Mortierella* was the most abundant genus (15.31%–21%) (Fig. 3b). At day 35, unclassified fungi in the “heavy” fraction samples increased and were the most abundant group (15.21%), followed by *Trichoderma* (12.69%). *Mortierella* remained the most abundant genus in both control treatments (15.63%–24.96%) (Fig. 3b). Analysis with FUNGuild assigned guilds to approximately 60% of the OTUs. The guild assignments demonstrated that all treatments, regardless of time point, were dominated by ectomycorrhizal fungi, soil saprotrophs and undefined saprotrophs (Fig. S6).

3.7. Co-occurrence network analyses

3.7.1. Heavy fraction

The co-occurrence network of “heavy” fraction samples (Fig. 4a) incorporated 59 nodes and 377 edges, with 51 bacterial nodes and 8 fungal nodes. At the genus level, 2 bacterial groups had connections with fungi, and 2 fungal groups were connected to bacteria. In total, 139 of the 377 connections were negative. The network contained several densely connected nodes, with 15 nodes having more than 20 neighbors each. The group with the highest number of connections was a *Planctomycetes* group, unclassified *Gemmataceae* (31 neighbors). Other groups with high connectivity were uncultured *Solirubrobacteraceae* (30 neighbors), unclassified *Armatimonadales* and *Acidothermus* (29 neighbors each). We also analyzed the bacterial genus identified as the most abundant in the “heavy” fractions, *Singulisphaera*, as well as the WH15EPS producer genus, *Granulicella*. In this treatment, *Singulisphaera* and *Granulicella* had 23 and 21 neighbors, respectively. Both genera had mainly positive connections (20 for *Singulisphaera* and 12 for *Granulicella*). No fungal group was connected to *Singulisphaera*, which was mainly connected to *Planctomycetes* and *Actinobacteria* (Fig. 4d). The groups associated with *Singulisphaera* and *Granulicella* are described in Supplementary Table S1. No strong associations were detected between *Granulicella* and *Singulisphaera*. The most abundant fungal genus, *Trichoderma*, did not have any strong correlation.

3.7.2. ¹²C-EPS-amended control

The network for the ¹²C-EPS-amended control (Fig. 4b) contained 29 nodes and 46 edges, with 18 bacterial nodes and 11 fungal nodes. Two bacterial groups were connected to fungi, and 3 fungal groups were connected to bacteria. No group had more than 10 neighbors, the maximum number of neighbors of a node in this network. *Singulisphaera* and uncultured *Pirellulaceae* had the highest number of neighbors (10), followed by *Rhodanobacter*, Unclassified KF-JG30-C25 and uncultured *Solirubrobacteraceae*, with 6 neighbors each. In this treatment,

Granulicella did not have any strong connections. *Singulisphaera* had only 2 negative connections, with *Dyella* and *Rhodanobacter* (Fig. 4e). The associations of *Singulisphaera* are described in Supplementary Table S2.

3.7.3. Unamended control

The network for the unamended control (Fig. 4c) possessed 54 nodes and 66 edges, with 41 bacterial and 13 fungal nodes. Among all associations, 2 bacterial groups were connected to fungi, and 3 fungal groups were connected to bacteria. No groups had more than 10 neighbors. Genus *Burkholderia-Caballeronia-Paraburkholderia* had the highest amount of neighbors (10), followed by *Conexibacter* (7 neighbors), *Dyella* and uncultured *Acidobacteriaceae* subgroup 1. (6 neighbors each). *Singulisphaera* had only one positive connection to unclassified *Iso-sphaeraceae* (Fig. 4f). *Granulicella* did not have any strong association.

4. Discussion

Here, we investigated the assimilation of EPS produced by *Granulicella* sp. strain WH15, a member of *Acidobacteria*. The ¹³C-labeled biopolymer was applied to organic topsoil samples, and high-throughput sequencing of the bacterial 16S rRNA gene and fungal ITS1 region identified the main EPS metabolizers. To increase the probability of finding true interactions, the organic topsoil samples were collected from the same forest site where *Granulicella* sp. strain WH15 was isolated (Valášková et al., 2009).

Although the degradation of bacterial-produced biopolymers has been explored previously (cellulose produced by the bacterium *Gluconacetobacter xylinus* (Verastegui et al., 2014; Wang et al., 2015), indican from *Beijerinckia indica* (Wang et al., 2015), and fructan from *Lactobacillus reuteri* (van Bueren et al., 2015)), the metabolism of EPS produced by *Acidobacteria* and its ecological implications have not been investigated. The addition of purified WH15EPS to the organic topsoil increased microbial activity compared with the unamended control, as measured by CO₂ respiration. The incorporation of the labeled EPS was confirmed by the increase in ¹³CO₂ release during incubation, which ranged from 10% to 43% of total headspace CO₂. The amount of ¹³CO₂ emitted varies according to the complexity of the substrate used for incubation and the capacity of the microbial community to degrade it (Štursová et al., 2012). Although the labeled material was clearly incorporated, the amount of “heavy” DNA recovered was lower than that reported in other SIP studies (Zhang et al., 2016), likely due to the complexity of the substrate, a heteropolysaccharide composed of 7 different monosaccharides (Kielak et al., 2017), and competition with the carbon present in the litter material (approximately 50% of organic matter). Longer incubation times can improve the recovery of “heavy” DNA; however, a limitation of this strategy is that it also increases the possibility of cross-feeder enrichment (Verastegui et al., 2014).

4.1. Alpha and beta diversity

The alpha diversity indices of the bacterial communities in the “heavy” fractions indicated lower richness and diversity than the amended and unamended controls at all sampling points, reflecting the selection of microorganisms capable of metabolizing the added EPS, particularly at day 35. These dynamics, however, were not as consistent for the fungal communities.

At day 8, the genus *Rhodanobacter* predominated in the EPS-amended control; however, the group unclassified *Pedospaeraceae* was the most abundant in the “heavy” fraction and in the unamended control. *Rhodanobacter* is a genus of the family *Xanthomonadaceae* (*Proteobacteria*) found in soils featuring decomposition of aromatic compounds (Nalin et al., 1999; Uhlik et al., 2012; Song et al., 2016) and forest litter (Štursová et al., 2012; Verastegui et al., 2014). *Pedospaeraceae* (subdivision 3) is an uncharacterized family within the phylum *Verrucomicrobia*, which, like *Acidobacteria*, is widespread among terrestrial environments and has few cultivated representatives (Spring

Fig. 4. Co-occurrence networks and conceptual framework of the interactions of microorganism during WH15EPS assimilation. Total networks for a) "heavy" fraction; b) Unlabeled EPS control; c) No EPS control (colors represent modularity classes); First neighbors of *Singulisphaera* genus in each treatment: d) "heavy" fraction; e) Unlabeled EPS control; f) No EPS control (network nodes are genera). The size of the nodes represents the number of neighbors. Black edges represent positive correlations; red edges represent negative correlations. The size of each node is proportional to the number of connections (degree); g) Cartoon illustrating simplified hypothetical relationships among microorganisms during the metabolization of the EPS produced by *Granulicella* sp strain WH15. *Singulisphaera* genus possessed negative (right (-)) correlations, as well as positive correlations (left (+)) with other bacterial genera. Negative associations suggest competition for the substrate, while positive associations suggest mutualism, via exchange of nutrients. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

et al., 2016).

Among the fungal communities, the genus *Scleroderma* was the most abundant in the “heavy” fractions and the second most dominant in the control samples after the genus *Mortierella* (which was also present in the heavy fraction). *Scleroderma* is a common, widespread ectomycorrhizal genus that produces macroscopic sporocarps in leaf litter, grass, bare soil or soil adjacent to forests (Jeffries, 1999). *Mortierella* is a root-colonizing endophytic fungus. Members of this globally distributed genus live as saprobes in soil on decaying organic material and dominate fungal communities in natural ecosystems (Johnson et al., 2018).

The significant differences in the bacterial and fungal communities among all treatments were most evident at the later time points, and therefore enrichment of any specific microbial genus in the “heavy” fraction could not be observed at day 8. Several genera and phyla appeared to incorporate WH15EPS, possibly due to the ready hydrolysis of the biopolymer by exoenzymes already present in the litter material at the time of incubation.

At day 24, in the bacterial communities, clear enrichment of the phylum *Planctomycetes* could be observed in the “heavy” fraction samples, reaching approximately 60% of the total sequence number, with the genus *Singulisphaera* as the most abundant. At day 35, the enrichment of *Planctomycetes* persisted, with the genus *Singulisphaera* among the predominant genera but overcome by another group of *Planctomycetes*, uncultured *Pirellulaceae*. At the genus level, the total abundance of *Planctomycetes* was distributed among *Singulisphaera*, *Candidatus Nostocoida* and unclassified groups such as uncultured *Pirellulaceae* and “WD2101 soil group”. *Planctomycetes* inhabit a variety of environments, including aquatic and terrestrial habitats as well as extremely acidic environments (Schlesner, 1994; Wang et al., 2002; Ivanova and Dedysh, 2012; Faria et al., 2018). *Planctomycetes* are highly abundant and phylogenetically diverse, especially in acidic *Sphagnum*-dominated wetlands, where they can account for up to 54% of the total amount of 16S rRNA gene sequences. The pH in *Sphagnum*-dominated wetlands can be similar to our sampling location (pH 3.0), ranging from pH 3.7 to 4.2 (Moore et al., 2015). Members of this phylum have unusual features, such as invaginations of the cytoplasmic membrane (Wiegand et al., 2018). Although the functions of *Planctomycetes* are not clearly understood, they possess a wide range of hydrolytic capabilities, which would explain the promotion of their enrichment by the metabolization of the complex heteropolysaccharide WH15EPS. Using a transcriptome approach, Ivanova et al. (2017a) observed that cellulose, xylan, pectin and chitin induced responses by different groups of *Planctomycetes*. The genus *Singulisphaera* responded significantly to pectin and xylan amendment. Members of the order *Phycisphaerales* and genus *Zavarzinella* also responded to xylan, while group WD2101 were responsive to cellulose and chitin. Another study by Ivanova et al. (2017b) further supported the high glycolytic potential of *Planctomycetes*. In that study, a comparative genomic analysis of 4 members of the family *Isosphaeraceae*, namely *Isosphaera pallida*, *Singulisphaera acidiphila*, *Paludisphaera borealis* and the uncharacterized strain SH-PL62S, identified several CAZymes from major families (GH5, GH13, GH57) as well as potential α -mannosidase, α -rhamnosidase and glucuronyl hydrolase activities. In addition, several CAZymes not affiliated with currently recognized enzymes were found, demonstrating that these bacteria have the ability to use a wide range of natural carbohydrates and undiscovered glycolytic potential. Furthermore, *Singulisphaera acidiphila* is capable of hydrolyzing several polysaccharides, such as laminarin, pectin, chondroitin sulfate, aesculin, pullulan, lichenan, xylan and gelatin (Kulichevskaya et al., 2008). Similar to the current study, Wang et al. (2015) reported enrichment of *Planctomycetes* by indican EPS of *Beijerinckia indica*. Indican is a biopolymer composed of glucuronic acid, glucose and glycerol-manno-heptose, indicating that *Planctomycetes* can decompose complex biopolymers.

Another group found in higher proportions in the “heavy” fractions at days 24 and 35 was uncultured *Methylacidiphilaceae*. This group belongs to the phylum *Verrucomicrobia*, which has few cultivated

representatives but has been revealed to have hydrolytic capabilities in cultivation-independent studies. Martinez-Garcia et al. (2012) demonstrated that members of *Verrucomicrobia* have high hydrolytic potential and encode a wide spectrum of glycoside hydrolases, carbohydrate lyases and esterases in their genomes, indicating that they are well-equipped with enzymes for carbohydrate metabolism. Cardman et al. (2014) demonstrated that fluorescently labeled laminarin and xylan preferentially associated with *Verrucomicrobia* and proposed that this phylum is involved in polysaccharide hydrolysis.

Within the fungal communities, at day 24, an increase in the proportions of the genus *Trichoderma* and groups of unclassified fungi was observed in the “heavy” fraction compared with both the amended and unamended controls. *Trichoderma* is a genus of filamentous ascomycete fungi present in soils or growing on wood, bark and other fungi (mycoparasite) (Druzhinina et al., 2011). This genus is highly opportunistic and adaptable to several environments, with some strains applied for biocontrol of fungal phytopathogens. *Trichoderma reesei* is capable of decomposing woody and herbaceous materials and is an important industrial producer of hemicellulolytic enzymes (Martinez et al., 2008; Druzhinina et al., 2011).

As expected, the classification of fungal sequences in guilds in the current study revealed that the most abundant fungi were ectomycorrhizal (ECM) fungi and saprotrophs related to organic material decomposition (Valášková et al., 2009; Urbanová et al., 2015). Many species of ECM fungi act as decomposers based on the expression of extracellular enzymes (Bödeker et al., 2009). Burke et al. (2014) indicated that enzyme activity varies greatly among ECM fungi, with some species producing enzymes at levels equivalent to those of saprotrophic fungi.

4.2. Co-occurrence and active potential interactions

Co-inertia analysis revealed significant co-variance between the fungal and bacterial communities, suggesting overall relationships between kingdoms. However, the co-occurrence networks showed that the overall number of connections between microbes was higher in the “heavy” fraction (377) than in either control treatment. The number of fungal genera connected to bacterial genera was lowest in the “heavy” fraction co-occurrence network (2), indicating that potential direct interactions between kingdoms did not increase during the assimilation of the EPS. Shorter arrows in co-inertia analysis for the heavy fraction, however, indicated a stronger relationship between bacterial and fungal communities, which could be due to indirect exchange of metabolites and use of fungal byproducts. Nonetheless, the assimilation of the EPS induced an increase in the potential interactions particularly among bacterial taxa, as observed in the number of edges in the “heavy” fraction network (377). These dynamics were not observed in the EPS-amended control network; nevertheless, not all taxa in this treatment are directly involved in the metabolism of the biopolymer. In addition, *Singulisphaera* had fewer potential interactions, with a variety of microorganisms from different phyla that have either cellulolytic capacity or the ability to remove toxic compounds (Valášková et al., 2009; Kielak et al., 2016b). *Granulicella* did not show any strong connection with any taxa in both control treatments.

Fungi are thought to be the main players in the decomposition of recalcitrant materials such as lignocellulose, followed by bacterial decomposition of polysaccharides and polymeric compounds (Boer et al., 2005; Romaní et al., 2006). In the “heavy” fraction, however, only 8 fungal groups, such as Unclassified *Leotiomyces* and the white-rot genus *Hypholoma* were observed. The fungal groups were mainly positively connected to each other, and negatively with unclassified groups of *Planctomycetes*, indicating competition for the carbon resources derived from the EPS, which hypothesis is supported by the high glycolytic capacity observed in both groups of microorganisms.

The most abundant bacterial genus in the “heavy” fraction, *Singulisphaera*, had mostly positive connections, especially with other groups of *Planctomycetes*, *Actinobacteria* and *Verrucomicrobia*, suggesting potential

cooperation for metabolizing WH15EPS. Several studies demonstrate that those groups of bacteria have glycolytic and detoxifying capacities, producing several enzymes that could be involved in the degradation of the EPS and resource sharing (Martinez-Garcia et al., 2012; Uhlik et al., 2012; Ivanova et al., 2017b). In addition, no direct correlation was found between *Singulisphaera* and the EPS producer *Granulicella*, which suggests that the metabolism of WH15EPS by *Singulisphaera* and enrichment of the genus did not negatively impact the abundance of *Granulicella*. This might be because of high amount of EPS added in the microcosm experiment. By contrast, *Granulicella* had a negative correlation with groups of *Actinobacteria*, demonstrating a potential competition, where *Actinobacteria* are metabolizing WH15EPS and impacting negatively *Granulicella*. *Actinobacteria* are widely spread in the environment and play an essential role in carbon cycling, presenting a wide range of extracellular enzymes (Lacombe-Harvey et al., 2018). Furthermore, the presence of *Granulicella* in the heavy fraction demonstrates the capacity of the genus to use EPS as a carbon source; however, there is no experimental evidence that the producer strain is able to metabolize its own EPS. It must be noted that the analysis of co-occurrence networks derived from labeled and unlabeled EPS treatments demonstrated that the treatment based on unlabeled total DNA may not reflect real co-occurrence among microorganisms, suggesting this that type of analysis can yield misleading ecological inferences. Even though network analysis has been a widely used approach in ecology, it still lacks experimental validation, especially due to technical limitations, such as the complexity of microbial ecosystems and the high number of uncultivated microorganisms (Berry and Widder, 2014). It has been observed that connections demonstrated by microbial networks do not necessarily represent real interactions (Faust and Raes, 2016), but predictions of potential interactions, which can also be indirect, and that the results of those evaluations should be interpreted carefully. Nonetheless, despite these limitations, co-occurrence network analysis can still lead to innovative insights into the structure of microbial communities (Faust and Raes, 2016) and establish a fundamental basis for more mechanistic studies of *Acidobacteria* and other uncultivated microbes. For instance, as shown in our study, the detection of *Singulisphaera* as the most abundant genus in the labeled treatment, as well as its connections to other microorganisms as shown in the “heavy” fraction network would not be possible in studies solely based on total DNA, due to the low relative abundance of such bacteria.

Several bacterial and fungal taxa have the ability and potential to metabolize the EPS of *Granulicella* sp. strain WH15. It is not possible to quantify the amount of EPS produced only by this genus in the natural environment; however, our study revealed active potential interactions between microorganisms in their natural habitat when EPS was added as carbon source. In addition, the structure of the co-occurrence network of microorganisms able to metabolize EPS differed from those in the control treatments, particularly in unlabeled EPS control, demonstrating that hidden potential interactions can be unraveled by more specific metabolism studies. Finally, the number of potential associations with uncultured and unclassified taxa reinforces that further efforts are needed to characterize these groups to better understand their functions in the environment.

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

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