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Supplementary Materials

Title: Extraction of extracellular polymeric substances (EPS) from Red Soils (Ultisols)

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1. Materials and methods

1.1 Site description and soil sampling

Two soils of contrasting ecosystems and Fe content were selected for the study. Forest soil was sampled from a cedar forest (114°21′E, 30°1′N) located in the He Shengqiao town, Xianning city, Hubei province, China. The region is characterized by a subtropical climate, with an average annual temperature of 16.8 ℃ and precipitation of 1300 mm. Paddy soil was collected from a paddy field under a rice-rice cropping system from National Agro-Ecosystem Observation and Research Station (116°55′E, 28°15′N) in Yingtan city, Jiangxi province, China. This region is characterized by a typical subtropical monsoonal climate, with an average annual temperature of 17.6 ℃ and precipitation of 1785 mm. Both soils are derived from Quaternary red clay. The soils were sampled randomly from 0-20 cm depth with three replicates per site, then soil sample were homogeneous mixing to produce composite soils. Then the soil samples (forest soils and paddy soils) were sieved (2 mm) and divided into divided into two subsamples. One subsample was air dried and used to analyze soil properties, while the other subsample was incubated for extraction of EPS as described below.

1.2 Determination of soil characteristics

Soil pH was measured in triplicate in soil-water prepared at a ratio of 1: 2.5. Soil particle size distributions were determined using the laser diffraction method (Mastersizer 3000, Malvern, UK). The contents of organic carbon and total nitrogen were analyzed in triplicate with an elemental analyzer (Vario MICRO cube, Elementar, Germany). The free iron oxides (Fe_d) and amorphous iron oxides (Fe_o) were extracted

using dithionite-citrate-bicarbonate (DCB) solution and oxalic acid ammonium-oxalate solution (McKeague and Day 1966), respectively. The extracts were diluted and iron concentration was measured with an atomic absorption spectrophotometer (AA240FS, Varian, USA).

1.3 Soil incubation

Inevitably, artefacts can be caused by soil sampling and sieving, which may affect subsequent analysis. For example, soil sampling may cause cell lysis. Consequently, we may overestimate cell lysis if we do not include the incubation treatment. Thus, when we compared the extraction methods, the soils sample were incubated. Briefly, the subsample (100 g dry weight equivalent) were pre-incubated at 25 °C for 2 weeks to ensure microbial activity had stabilized caused by sampling, sieving and adjustment of soil moisture (e.g. Redmile-Gordon et al., 2014; Brookes et al., 2017). During the incubation period, the water content of the soils was kept at 60% of maximum water holding capacity (WHC). In order to maintain constant soil water content, deionized water was added gravimetrically twice weekly. After pre-incubation, microcosms were incubated under the same conditions but with an added substrate for a further 10 days as did Redmile-Gordon et al. (2014), since EPS production is understood to be the greatest sometime shortly after the exponential phase of growth. The exponential phase of growth in soils given additional substrate at these temperatures tends to last between 1 and 7 days. Hence, the time-point of 10 days was selected to ensure this phase had passed. The extra substrate was used to stimulate the microbial biomass to produce EPS (Nunan et al. 2003). Glycerol was

selected since it produced no sugars or proteins on decomposition (Redmile-Gordon et al. 2014), which could otherwise affect the quantification of components in EPS. The glycerol was added to soils at a concentration of 10 mg C g^{-1} soil. In addition, to ensure that the growth of soil microbes was not limited by the lack of nutrients, ammonium nitrate and mono-ammonium phosphate were added to soils at concentrations of 1.16 mg N g^{-1} soil and 0.166 mg P g^{-1} soil, respectively. We added C:N:P according to average global stoichiometric ratios of C:N:P in soil microbial biomass (60:7:1) (Cleveland and Liptzin, 2007).

1.4 Extraction of EPS

Before EPS extraction, soluble microbial products (SMP) were extracted from soils (Redmile-Gordon et al., 2014). Soluble microbial products (SMP) are freely soluble extracellular polymers not actually bound to the cells or EPS matrix (see Comte et al., 2006). The transient nature of SMP means that it is not a defining component of the extracelluar matrix or 'biofilm'. Therefore, any extraction method should include SMP. To extract SMP, 25 mL pre-cooled $CaCl_2$ solution (pH 7.0, 10 mM) was added to the moist soil (2.5 g dry weight equivalent), shaken at 120 rpm (4 °C) for 30 min, then centrifuged at 4000 g for 30 min (4 °C). EPS was subsequently extracted from the residues with the followin five method.

1.4.1 Hot water extractable polysaccharide (HWEP)

The HWEP extraction methods were slightly modified from the method described by Ghani et al. (2003). This method were frequently used to extract EPS EPS-like fraction (e.g. polysaccharides) from soils. Briefly, 25 mL of ultra-pure water

was added to the residues (after extraction of SMP), incubated in a water bath (80 °C) for 7 h, then centrifuged at 4000 g for 30 min (4 °C). The supernatant was passed through 0.45 μm cellulose nitrate membrane filters and then was used for EPS determination (within 4 days). The pellet was washed using phosphate buffer saline (PBS), centrifuged at 4000 g for 30 min and the supernatant of PBS was discarded, then the pellet was stored at -80 °C pending ATP analysis.

1.4.2 Hot dilute acid extractable polysaccharide (HDAEP) extraction

The HWEP extraction methods were slightly modified from the method described by Spohn and Giani (2010). This method were frequently used to extract EPS-like fraction (e.g. polysaccharides) from soils. Briefly, 25 mL of dilute sulfuric acid (0.125 M H_2SO_4) was added to the residues (after extraction of SMP), incubated in a water bath (80 °C) for 7 h, then centrifuged at 4000 g for 30 min (4 °C). The supernatant was passed through 0.45 μm cellulose nitrate membrane filters and then was used for EPS determination (within 4 days). The pellet was washed using phosphate buffer saline (PBS), centrifuged at 4000 g for 30 min and the supernatant of PBS was discarded, then the pellet was stored at -80 °C pending ATP analysis.

1.4.3 Easily extractable glomalin (EEG) extraction

The EEG protocol was first developed by Wright and Upadhyaya (1996) to extract a protein (i.e. glomalin) deposited by arbuscular mycorrhizal fungi (AMF). The protocol described by Wright and Upadhyaya (1996) was followed. Briefly, 20 mL of 20 mM citrate buffer (pH 7.0) was added to the residues (after extraction of SMP), autoclaved at 121 °C for 30 min, then centrifuged at 4000 g for 30 min (4 °C).

111 The supernatant was passed through 0.45 μm cellulose nitrate membrane filters and
112 then was used for EPS determination (within 4 days). The pellet was washed using
113 phosphate buffer saline (PBS), centrifuged at 4000 g for 30 min and the supernatant
114 of PBS was discarded, then the pellet was stored at -80 °C pending ATP analysis.

115 **1.4.4 Sodium sulfide extraction**

116 The theory of EPS extraction with SS is that SS can reduce the Fe^{3+} to insoluble
117 FeS, thus resulting in disintegration of Fe^{3+} bound EPS matrix Nielsen and Keiding
118 (1998). The extraction procedure was slightly modified from the method described by
119 Zhu et al. (2015). Briefly, 25 mL of 20 mM sodium sulfide ($Na_2S{\cdot}9H_2O$) was added to
120 the residues (after extraction of SMP), shaken at 180 rpm (4 °C) for 2 h, then
121 centrifuged at 4000 g for 30 min. The supernatant was passed through 0.45 μm
122 cellulose nitrate membrane filters and then was used for EPS determination (within 4
123 days). The pellet was washed using phosphate buffer saline (PBS), centrifuged at
124 4000 g for 30 min and the supernatant of PBS was discarded, then the pellet was
125 stored at -80 °C pending ATP analysis.

126 **1.4.5 Cation exchange resin (CER) extraction**

127 Extraction buffer was prepared as 4 mM NaH_2PO_4, 2 mM Na_3PO_4, 9 mM NaCl
128 and 1 mM KCl at pH 7.0 (Frolund et al. 1996). CER (91973, Sigma-Aldrich) was
129 pretreated for a pH of 7.0 and was washed three times with the EPS extraction buffer
130 before use. The CER was added at the amount of 70 g CER g^{-1} volatile solids (VS),
131 and the quantity of VS in the soil was determined according to Redmile-Gordon et al.
132 (2014). 70 g CER g^{-1} volatile solids (VS) was widely used in EPS extraction from

133 sludge (e.g. Frolund et al., 1996), since EPS can be extracted with maximum
134 efficiency with this amount CER. The theory of EPS extraction with CER is that a
135 combination of shear forces and resin-Na^+ cause ion exchange with multivalent
136 cations (mainly Ca^{2+} and Mg^{2+}) that link EPS resulting in dissolution of EPS
137 macrostructure (Wilén et al., 2003).

138 To extract EPS, 25 mL of the pre-cooled extraction buffer and pre-weighed CER
139 were added to the residues (after extraction of SMP), shaken at 180 rpm (4 °C) for 2 h,
140 then centrifuged at 4000 g for 30 min. The supernatant was passed through 0.45 μm
141 cellulose nitrate membrane filters and then was used for EPS determination (within 4
142 days). The pellet was washed using phosphate buffer saline (PBS), centrifuged at
143 4000 g for 30 min and the supernatant of PBS was discarded, then the pellet was
144 stored at -80 °C pending ATP analysis.

145 **1.5 Characterization of EPS**

146 The polysaccharide content was determined by the anthrone-sulfuric acid method
147 (Brink Jr et al., 1960) using glucose (G116307, Aladdin) as the standard. The protein
148 content was estimated using the Bradford method (Bradford, 1976) with bovine serum
149 albumin (A104912, Aladdin) as the standard. The HAE content was measured using
150 the method described by Wang and Fujii (2011) using humic acid as the standard
151 (H16752, Sigma-Aldrich). All of these components of EPS were quantified by
152 UV-visible spectroscopy (A580, Ao Yi Instrument CO. LED., China).

153 **1.6 Measurement of ATP content of soils before and after EPS extraction**

154 ATP was extracted from soils (without EPS extraction) and the pellet (after EPS

155 extraction) using the method of Redmile-Gordon et al. (2011) with Extractant A and B.
156 Extractant A contained 1.1 M trichloroacetic acid, 0.6 M imidazole, 0.25 M sodium
157 hydrogen phosphate. Extractant B was similar to Extractant A, except that it contained
158 5 mL added 0.1 mM ATP. During extraction, some of the ATP may be sorbed on soil
159 colloids, or denatured. This was corrected for by determining the proportion of added
160 ATP recovered in soil Extract B and using this percentage recovery to correct for the
161 same loss processes in soil during extraction with soil Extractant A. Briefly, moist
162 soils or the pellet with 25 ml Extractant A or B were ultrasonified for 2 min, cooled on
163 ice for 5 min, then filtered (Whatman 42).

164 The analysis of ATP as described by Qiu et al. (2016). Briefly, 10 μL extract was
165 added to 150 μL arsenate buffer, 13 μL 1M NaOH, and 50 μL luciferin-luciferase
166 (GN202-01, Beijing yuanpinghao biotechnology co. LTD, China) and the
167 bioluminescence of the mixture was measured using a Multimode Plate Reader
168 (EnVision, PerkinElmer, USA) in dark 96-well microplates.

169 **1.7 Statistical analyses**

170 The statistical analysis was conducted with SPSS 23.0. We check normality and
171 homoscedasticity of variances by ShapiroeWilks test and Levene's test, respectively.
172 In some cases, data was log transformed to meet the normal distribution. An analysis
173 of variance (ANOVA) followed by Duncan’s multiple-comparison test at $p < 0.05$ was
174 used to evaluate the differences between EPS content extracted by different methods
175 in the same soil. Student’s t-tests were used to evaluate the statistical significance of
176 differences between means, of 1) the content of C, N and iron oxides in the two soils,

177 and 2) soil microbial ATP content in the same soil before and after EPS extraction.

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Table S1 Physical and chemical properties of the two soils. Data (means ± SE, n = 3) followed by the different letters within a column indicate significant differences ($p < 0.05$).

Soil	Clay (%)	Silt (%)	Sand (%)	pH	SOC (g kg^{-1})	TN (g kg^{-1})	C/N ratio	Fe_d (g kg^{-1})	Fe_o (g kg^{-1})
Forest soil	18.5±0.5	79.6±0.6	1.9±0.2	5.6	8.75±0.15b	0.86±0.33b	9.9±0.3b	29.4±0.5a	2.53±0.07a
Paddy soil	15.6±0.2	61.7±0.7	22.7±0.9	4.7	21.66±0.58a	1.84±0.05a	11.8±0.1a	6.8±0.8b	1.64±0.03b

Abbreviations: SOC, soil organic carbon; TN, total nitrogen; Fe_d, dithionite-citrate-bicarbonate extractable iron oxides; Fe_o, oxalate extractable iron oxides.