



UNIVERSITY OF LEEDS

This is a repository copy of *Extraction of extracellular polymeric substances (EPS) from red soils (Ultisols)*.

White Rose Research Online URL for this paper:
<http://eprints.whiterose.ac.uk/146966/>

Version: Supplemental Material

Article:

Wang, S, Redmile-Gordon, M, Mortimer, M et al. (5 more authors) (2019) Extraction of extracellular polymeric substances (EPS) from red soils (Ultisols). *Soil Biology and Biochemistry*, 135. pp. 283-285. ISSN 0038-0717

<https://doi.org/10.1016/j.soilbio.2019.05.014>

© 2019 Elsevier Ltd. Licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Reuse

This article is distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives (CC BY-NC-ND) licence. This licence only allows you to download this work and share it with others as long as you credit the authors, but you can't change the article in any way or use it commercially. More information and the full terms of the licence here: <https://creativecommons.org/licenses/>

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.



eprints@whiterose.ac.uk
<https://eprints.whiterose.ac.uk/>

1 **Supplementary Materials**

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

3 Authors: Shuang Wang¹, Marc Redmile-Gordon², Monika Mortimer³, Peng Cai^{1, *},

4 Caroline L. Peacock⁴, Yichao Wu¹, Chunhui Gao¹, Qiaoyun Huang¹

5

6 ¹State Key Laboratory of Agricultural Microbiology, College of Resources and

7 Environment, Huazhong Agricultural University, Wuhan 430070, China

8

9 ²Department of Environmental Horticulture, Royal Horticultural Society, Wisley,

10 Surrey, GU23 6QB, UK

11

12 ³Bren School of Environmental Science and Management, Earth Research Institute

13 and University of California Center for the Environmental Implications of

14 Nanotechnology (UC CEIN), University of California, Santa Barbara, California

15 93106, United States

16

17 ⁴School of Earth and Environment, University of Leeds, Leeds LS2 9JT, UK

18

19 * Corresponding author. Phone: +86 27 87671033; Fax: +86 27 87280670

20 E-mail address: cp@mail.hzau.edu.cn (P. Cai)

21

22

23 **1. Materials and methods**

24 **1.1 Site description and soil sampling**

25 Two soils of contrasting ecosystems and Fe content were selected for the study.
26 Forest soil was sampled from a cedar forest (114°21'E, 30°1'N) located in the He
27 Shengqiao town, Xianning city, Hubei province, China. The region is characterized by
28 a subtropical climate, with an average annual temperature of 16.8 °C and precipitation
29 of 1300 mm. Paddy soil was collected from a paddy field under a rice-rice cropping
30 system from National Agro-Ecosystem Observation and Research Station (116°55'E,
31 28°15'N) in Yingtan city, Jiangxi province, China. This region is characterized by a
32 typical subtropical monsoonal climate, with an average annual temperature of 17.6 °C
33 and precipitation of 1785 mm. Both soils are derived from Quaternary red clay. The
34 soils were sampled randomly from 0-20 cm depth with three replicates per site, then
35 soil sample were homogeneous mixing to produce composite soils. Then the soil
36 samples (forest soils and paddy soils) were sieved (2 mm) and divided into divided
37 into two subsamples. One subsample was air dried and used to analyze soil properties,
38 while the other subsample was incubated for extraction of EPS as described below.

39 **1.2 Determination of soil characteristics**

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

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

49 **1.3 Soil incubation**

50 Inevitably, artefacts can be caused by soil sampling and sieving, which may
51 affect subsequent analysis. For example, soil sampling may cause cell lysis.
52 Consequently, we may overestimate cell lysis if we do not include the incubation
53 treatment. Thus, when we compared the extraction methods, the soils sample were
54 incubated. Briefly, the subsample (100 g dry weight equivalent) were pre-incubated at
55 25 °C for 2 weeks to ensure microbial activity had stabilized caused by sampling,
56 sieving and adjustment of soil moisture (e.g. Redmile-Gordon et al., 2014; Brookes et
57 al., 2017). During the incubation period, the water content of the soils was kept at
58 60% of maximum water holding capacity (WHC). In order to maintain constant soil
59 water content, deionized water was added gravimetrically twice weekly. After
60 pre-incubation, microcosms were incubated under the same conditions but with an
61 added substrate for a further 10 days as did Redmile-Gordon et al. (2014), since EPS
62 production is understood to be the greatest sometime shortly after the exponential
63 phase of growth. The exponential phase of growth in soils given additional substrate
64 at these temperatures tends to last between 1 and 7 days. Hence, the time-point of 10
65 days was selected to ensure this phase had passed. The extra substrate was used to
66 stimulate the microbial biomass to produce EPS (Nunan et al. 2003). Glycerol was

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

75 **1.4 Extraction of EPS**

76 Before EPS extraction, soluble microbial products (SMP) were extracted from
77 soils (Redmile-Gordon et al., 2014). Soluble microbial products (SMP) are freely
78 soluble extracellular polymers not actually bound to the cells or EPS matrix (see
79 Comte et al., 2006). The transient nature of SMP means that it is not a defining
80 component of the extracellular matrix or 'biofilm'. Therefore, any extraction method
81 should include SMP. To extract SMP, 25 mL pre-cooled CaCl₂ solution (pH 7.0, 10
82 mM) was added to the moist soil (2.5 g dry weight equivalent), shaken at 120 rpm
83 (4 °C) for 30 min, then centrifuged at 4000 g for 30 min (4 °C). EPS was
84 subsequently extracted from the residues with the following five methods.

85 **1.4.1 Hot water extractable polysaccharide (HWEPS)**

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

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

95 **1.4.2 Hot dilute acid extractable polysaccharide (HDAEP) extraction**

96 The HWEP extraction methods were slightly modified from the method
97 described by Spohn and Giani (2010). This method were frequently used to extract
98 EPS-like fraction (e.g. polysaccharides) from soils. Briefly, 25 mL of dilute sulfuric
99 acid (0.125 M H₂SO₄) was added to the residues (after extraction of SMP), incubated
100 in a water bath (80 °C) for 7 h, then centrifuged at 4000 g for 30 min (4 °C). The
101 supernatant was passed through 0.45 µm cellulose nitrate membrane filters and then
102 was used for EPS determination (within 4 days). The pellet was washed using
103 phosphate buffer saline (PBS), centrifuged at 4000 g for 30 min and the supernatant
104 of PBS was discarded, then the pellet was stored at -80 °C pending ATP analysis.

105 **1.4.3 Easily extractable glomalin (EEG) extraction**

106 The EEG protocol was first developed by Wright and Upadhyaya (1996) to
107 extract a protein (i.e. glomalin) deposited by arbuscular mycorrhizal fungi (AMF).
108 The protocol described by Wright and Upadhyaya (1996) was followed. Briefly, 20
109 mL of 20 mM citrate buffer (pH 7.0) was added to the residues (after extraction of
110 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 $-80\text{ }^{\circ}\text{C}$ 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 ($\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$) was added to
120 the residues (after extraction of SMP), shaken at 180 rpm ($4\text{ }^{\circ}\text{C}$) 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 $-80\text{ }^{\circ}\text{C}$ 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 μ L extract was
165 added to 150 μ L arsenate buffer, 13 μ L 1M NaOH, and 50 μ L 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 Shapiro-Wilks 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.

178 **References**

179 Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of
180 microgram quantities of protein utilizing the principle of protein-dye binding.

181 *Analytical Biochemistry* 72, 248–254.

182 Brink Jr, R.H., Dubach, P., Lynch, D.L., 1960. Measurement of carbohydrates in soil
183 hydrolyzates with anthrone. *Soil Science* 89, 157–166.

184 Cleveland, C.C., Liptzin, D., 2007. C: N: P stoichiometry in soil: is there a “Redfield
185 ratio” for the microbial biomass?. *Biogeochemistry* 85, 235–252.

186 Comte, S., Guibaud, G., Baudu, M., 2006. Biosorption properties of extracellular
187 polymeric substances (EPS) resulting from activated sludge according to their
188 type: soluble or bound. *Process Biochemistry* 41, 815–823.

189 Filisetti-Cozzi, T.M., Carpita, N.C., 1991. Measurement of uronic acids without
190 interference from neutral sugars. *Analytical Biochemistry* 197, 157–162.

191 Ghani, A., Dexter, M., Perrott, K.W., 2003. Hot-water extractable carbon in soils: a
192 sensitive measurement for determining impacts of fertilisation, grazing and
193 cultivation. *Soil Biology and Biochemistry* 35, 1231–1243.

194 McKeague, J.A., Day, J., 1966. Dithionite-and oxalate-extractable Fe and Al as aids in
195 differentiating various classes of soils. *Canadian Journal of Soil Science* 46,
196 13–22.

197 Nunan, N., Wu, K.J., Young, I.M., Crawford, J.W., Ritz, K., 2003. Spatial distribution

198 of bacterial communities and their relationships with the micro-architecture of
199 soil. *FEMS Microbiology Ecology* 44, 203–215.

200 Qiu, G.Y., Chen, Y.F., Luo, Y., Xu, J.M., Brookes, P.C., 2016. The microbial ATP
201 concentration in aerobic and anaerobic Chinese soils. *Soil Biology and*
202 *Biochemistry* 92, 38–40.

203 Redmile-Gordon, M.A., White, R.P., Brookes, P.C., 2011. Evaluation of substitutes
204 for paraquat in soil microbial ATP determinations using the trichloroacetic acid
205 based reagent of Jenkinson and Oades (1979). *Soil Biology and Biochemistry* 43,
206 1098–1100.

207 Redmile-Gordon, M.A., Brookes, P.C., Evershed, R.P., Goulding, K.W.T., Hirsch, P.R.,
208 2014. Measuring the soil-microbial interface: Extraction of extracellular
209 polymeric substances (EPS) from soil biofilms. *Soil Biology and Biochemistry*
210 72, 163–171.

211 Spohn, M., Giani, L., 2010. Water-stable aggregates, glomalin-related soil protein,
212 and carbohydrates in a chronosequence of sandy hydromorphic soils. *Soil*
213 *Biology and Biochemistry* 42, 1505–1511.

214 Wright, S.F., Upadhyaya, A., 1996. Extraction of an abundant and unusual protein
215 from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi.
216 *Soil Science* 161, 575–586.

217 Wang, Y., Fujii, T., 2011. Evaluation of methods of determining humic acids in
218 nucleic acid samples for molecular biological analysis. *Bioscience*

- 219 Biotechnology and Biochemistry 75, 355–357.
- 220 Zhu, L., Yu, H.T., Liu, Y.M., Qi, H.Y., Xu, X.Y., 2015. Optimization for extracellular
221 polymeric substances extraction of microbial aggregates. Water Science and
222 Technology 71, 1106–1112.

223 **Table S1** Physical and chemical properties of the two soils. Data (means \pm SE, n = 3) followed by the different letters within a column indicate
224 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 \pm 0.5	79.6 \pm 0.6	1.9 \pm 0.2	5.6	8.75 \pm 0.15b	0.86 \pm 0.33b	9.9 \pm 0.3b	29.4 \pm 0.5a	2.53 \pm 0.07a
Paddy soil	15.6 \pm 0.2	61.7 \pm 0.7	22.7 \pm 0.9	4.7	21.66 \pm 0.58a	1.84 \pm 0.05a	11.8 \pm 0.1a	6.8 \pm 0.8b	1.64 \pm 0.03b

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