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Measuring the soil-microbial interface: Extraction of extracellular polymeric substances (EPS) from soil biofilms☆

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ABSTRACT

Many soil microbes exist in biofilms. These biofilms are typified by variable quantities of extracellular polymeric substances (EPS: predominantly polysaccharides, glycoconjugates, and proteins) and the embedded microbial cells. A method to measure soil-EPS (the biofilm exclusive of microbial cells) has not yet been described. The present work investigates the potential of five extraction methods to estimate changes in soil-EPS content. A rationale for selection of appropriate EPS extraction and methodology is discussed, including the crucial consideration of both intracellular and extracellular contamination.

EPS was developed *in situ* by provision of labile C (glycerol) to the microbial biomass of a moist soil and then applying desiccation stress. Only two out of the five extraction methods showed statistically significant increases in polysaccharide production responding to substrate addition. Humified organic matter, estimated by its humic acid equivalent (HAE) was used to indicate the degree of extracellular contamination, and/or creation of humic artefacts – both of which affect detection of changes in EPS. The HAE concentration was very high when applying original and modified methods designed to extract glomalin related soil protein (GRSP). Extraction methods involving heating with dilute sulphuric acid appeared to overestimate EPS-polysaccharide. Using microbial ATP as an indicator of cell-lysis, confidence could only be ascribed to EPS extraction with cation exchange resin. Using this method, the expected increases in EPS-polysaccharide were clearly apparent. The HAE/protein ratios of EPS extracts were also lowest with cation exchange – indicating this method did not cause excessive contamination from humified soil organic matter or create related artefacts.

1. Introduction

1.1. The soil–microbial interface

A range of techniques has been developed to help estimate the soil microbial mass, such as soil biomass C (Vance et al., 1987) and ATP (Jenkinson and Oades, 1979). These bioindicators have long been used to increase our understanding of the connection between soil microbial communities and soil functions, e.g. helping to predict changes in soil organic matter (Powlson et al., 1987). The microbial biomass (specifically the cell content) is recognised to contribute towards indices of soil quality (e.g. Ritz et al., 2009) because soil biology drives key processes of value to the global economy (Haygarth and Ritz, 2009). However, until now, work to characterise this microbial mass has focussed upon the *intracellular* content, whereas over 99% of microbial life on earth is thought to exist in *biofilms* (Vu et al., 2009). Biofilms are aggregates of microbes connected by extracellular polymeric substance, or 'EPS'. The EPS is exported from the intracellular space, to form an extracellular polymeric matrix (Flemming and Wingender, 2010) with the EPS accounting for variable proportions, comprising up to 80% of a soil biofilm's dry mass (Chenu, 1993).

Understanding of biofilms in the aquatic and engineering sciences is comparatively advanced. Competitive advantage for microbial life is known to be achieved through EPS production, which improves quorum sensing (Elias and Banin, 2012), colony adhesion (Flemming and Wingender, 2001), syntrophy (Bernstein et al., 2012), defence against predation (Decho and Lopez, 1993), desiccation tolerance and solute transport (Roberson and Firestone, 1992; Chenu and Roberson, 1996), and tolerance to heavy metals (Yang et al., 2013). The EPS also provides a template for extracellular

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enzymes in aquatic systems, preventing enzymes from loss and prolonging the duration of their activity (Romani et al., 2008; Pohlon et al., 2013). EPS can even facilitate cell-movement, described as 'bacterial gliding' (Hu et al., 2012). Other scientific disciplines have evidently revealed a wealth of knowledge or hypotheses that stand untested in soil science. A better understanding of these active films in soil is central to our better management of biologically mediated nutrient turnover and soil health generally (Burns et al., 2013).

EPS produced *in situ* is expected to also improve soil properties, for example, increasing heterogeneity (Davis et al., 2009) and improving soil aggregate stability (Spohn and Giani, 2010; Tang et al., 2011). Agronomically, the inoculation of wheat seedlings with EPS-producing *Bacillus* and *Enterobacter* species was shown to afford saline tolerance (Upadhyay et al., 2011), and survival rates of sunflowers in drought were improved by inoculation with EPS-producing strains of *Pseudomonas* (Sandhya et al., 2009).

Young and Crawford (2004) introduced the concept that the soil–microbe system is 'self-organising'. The evolution of microbial life is suspected to be strongly influenced by hydration cycles affecting connectivity, and, in turn, evolving systems are likely to modify this connectivity (Crawford et al., 2005). An evolutionary framework for soil ecology and microstructure is still lacking, and the ability to manage soil via understanding of this framework remains unexplored (Crawford et al., 2012). Since a method to measure EPS is central to this framework, it is clearly an important goal towards achieving sustainable agriculture and food security. Water deficit in soils is the most damaging abiotic environmental stress affecting agricultural productivity worldwide (Hanjra and Qureshi, 2010). The impact of EPS on hydrology is a salient point, since the polymers exhibit hydrophilic and hydrophobic moieties, with EPS imparting both slowed wettability, and slowed 'de-watering' properties to the surrounding porous media (Or et al., 2007a). EPS can hold up to 20 times its own weight in water (Or et al., 2007b), but a method to measure EPS in soil has not yet been established.

Investigations of mixed-species EPS using confocal laser scanning microscopy (CLSM) combined with lectin-binding techniques have been invaluable for characterisation of biofilms in culture, marine, and freshwater systems (Neu and Lawrence, 1999; Staudt et al., 2003; Zippel and Neu, 2011). However, revealing mixed-species EPS in soil is extremely challenging due to i) physical obstruction by mineral particles ii) influence of sample preparation, and iii) the typical abundance of humic compounds and decaying plant-carbohydrates which can interfere with the binding of fluorescent labels (Thomas Neu, *pers comm*). Nonetheless, the existence of biofilms in soil -and hence EPS- is strongly suggested by the highly ordered and patchy distributions of microbial cells, which are more easily visualised, existing in clusters, especially where substrate availability is high (Nunan et al., 2003). The present work investigates the potential of several extraction approaches to measure changes in the quantity of EPS assumed to be produced in soil.

1.1.1. Difficulties extracting EPS: intracellular contamination

The EPS of mixed-species biofilms is primarily composed of carbohydrates, proteins, and uronic acids, with smaller quantities of DNA and glycolipids (Flemming and Wingender, 2010). Negatively charged moieties are typically abundant, with multivalent cations linking glycoconjugates of the EPS matrix (Frolund et al., 1996; Sheng et al., 2010). The greatest problem with extracting EPS, occurs when methods are too harsh, where intracellular material is released into the extract (Flemming and Wingender, 2010). Hence this aspect is typically validated (or not) depending upon the confidence given by a measure of cell-lysis (Liu and Fang, 2002).

Frolund et al. (1996) used DAPI staining of intact cells that remained after the EPS extraction was complete, but the abundance of opaque and reflective mineral surfaces make this approach more difficult in soil. Both DNA and ATP measurements have previously been used as indicators of lysis (Takahashi et al., 2009). Recently however, it has been acknowledged that DNA is an integral component of the EPS matrix itself (Cheng et al., 2011). Dominiak et al. (2011) found that ammonium-oxidising *Nitrosomonas*, and nitrite-oxidising *Nitrospira* exported disproportionately large quantities of DNA to the EPS, almost 300 mg of extracellular DNA were detected per gram of EPS. Subsequent digestion of the EPS with DNase caused disintegration of microcolonies with high extracellular DNA concentrations, suggesting that extracellular DNA can be an important structural component. Furthermore, Pote et al. (2010) found that, on average, between 20 and 50% of the total DNA in lake sediments was present as extracellular DNA. Therefore, the DNA quantity in extracts cannot reliably be used as a measure of lysis.

The quantity of ATP hydrolysed during extraction is a more promising indicator of the extent of lysis, because ATP has a very short half-life in soil, owing to the typical abundance of phosphatases (e.g. Renella et al., 2002). Although a small amount of soil-native ATP has been seen to resist enzymatic hydrolysis by attachment to clay surfaces, this fraction does not interfere with measurements (Cowan and Casanueva, 2007). The quantity of extracted ATP can therefore indicate the amount of intact cellular biomass, and therefore -by difference- provide a measure of cell-lysis caused by EPS extraction. Besides estimating lysis, microbial ATP can also provide an alternative measure of the microbial biomass in soils where the C content may otherwise be confounding (Joergensen, 1995), specifically with soils recently given carbon-rich substrates (Joergensen and Raubuch, 2002; Luo et al., 2013), and a surplus of labile C is suspected to be required for significant production of EPS in soil (Nunan et al., 2003).

1.1.2. Difficulties extracting EPS: extracellular contamination

In water technology, biofilms are sometimes considered as being the collection of cells and '*all other external organic matter*' (e.g. McSwain et al., 2005). This 2-part definition is not suitable for investigations in soil because distinction is also required between the biofilm and the decomposing organic biochemicals and humic substances which are understood to form the bulk of organic matter in mineral soils (Piccolo, 2002). It is therefore apparent that at least three pools of soil organic matter require consideration when comparing approaches for soil biofilm extraction: 1) cells of the microbial biomass 2) the EPS itself, and 3) non-biofilm soil organic matter (NBSOM). When measuring EPS it is important to avoid techniques which co-extract large amounts of NBSOM to prevent subsequent misinterpretations. For this reason EPS extraction techniques should aim to be conservative, rather than exhaustive. Estimating the quantity of co-extracted humified organics is no simple task because by definition these substances lack clear biochemical structure (Baldock and Nelson, 2000; Piccolo, 2002; Kleber and Johnson, 2010). Colorimetric methods are commonly applied to estimate the humic fraction in water sciences (e.g. Liu and Fang, 2002) and also to estimate the amount of humic interference in protein measurements (previously expressed as humic-acid equivalents or 'HAE'; Redmile-Gordon et al., 2013). Since no humic substances were found in EPS extracted from a range of pure microbial cultures grown in vitro (Guibaud et al., 2005) the concentration of HAE is expected to provide a useful indicator of non-specific extraction and/or humic artefacts generated by extraction processes.

1.2. Current extraction methods

In water technology, Liu and Fang (2003) observed that the plethora of methods in use to extract EPS at that time made comparisons between studies difficult. Many subsequent comparisons described problems associated with extractants, heating, or contamination by lysis of the microbial biomass (Comte et al., 2006b). More recently, extraction of EPS using cation exchange resin (CER) has become more popular than the other more exhaustive approaches (Sheng et al., 2010) owing to i) minimal change of original EPS chemical structures (Comte et al., 2006b), ii) high extraction efficiency for protein (D'Abzac et al., 2010), and iii) propensity to maintain cell-integrity during extraction (Takahashi et al., 2009).

In soils, there has been no method described previously to extract EPS *per se*, but there has been a great amount of work in seeking to quantify 'glomalin related soil protein' ('GRSP'). This follows discovery of a protein deposited extracellularly by arbuscular mycorrhizal fungi (AMF) and development of an extraction protocol for this substance, named Glomalin by Wright and Upadhyaya (1996). Operationally defined 'GRSP' shows strong correlations to a variety of soil properties, from carbon storage to aggregate stability (Rillig et al., 2001). However, applying proteomics, a comprehensive investigation by Gillespie et al. (2011) showed this pool of SOM contained very little material likely to be of AMF origin, but rather proteins likely to originate from free-living soil bacteria. Both AMF and EPS are thought to be important for agriculture: AMF increasing root surface area for water and nutrient uptake (Smith and Smith, 2011) and EPS retaining water in soils prone to drought (Rosenzweig et al., 2012). However, 'GRSP' has also been seen to increase in response to moderate water stress (Kohler et al., 2009). Furthermore, 'GRSP' content is not always linked to the abundance of AM-fungi (e.g. Feeney et al., 2004) but was spatially associated with carbohydrate content (Wu et al., 2012) and enzyme activity (Bai et al., 2009). This suggests that extracted 'GRSP' could be more representative of microbial EPS than of Glomalin. Another extraction technique, commonly termed *weak acid extractable polysaccharide* (WAEP) was recently used to estimate EPS-polysaccharide (Tang et al., 2011). However, as with the GRSP method, the original WAEP method was not originally designed to extract extracellular material *per se* (Lowe, 1994). Nevertheless, the increasing attention received by this method justifies its evaluation.

Or et al. (2007a) suggested 'hydraulic decoupling' was a key feature of EPS, where, besides protecting cells against osmotic stress, the EPS buffered against rapid fluctuations in hydration status of the surrounding porous matrix. Besides the relevance for soil function, this also has important implications for extraction, i.e. when a dry matrix (the soil) is first saturated with extractant, EPS will initially resist the aqueous influx, and so hydraulic decoupling is likely to affect the extraction efficiency from air-dry soil. We therefore include modified versions of the soil methods described above, with addition of a 'hydraulic coupling' step, which allows the EPS to reach hydraulic equilibrium with the extractants. Our aim was to compare the aforementioned techniques, and identify the most promising protocol. In this study, EPS is considered as the measurable increase in extracellular biopolymers (polysaccharide, protein and uronic acid) extracted from heterotrophic soil biofilms developing in response to provision of labile substrate (glycerol).

2. Materials and methods

2.1. Site description, soil sampling and preparation

Two soils were selected with the same mineral parentage but contrasting managements leading to very different SOM contents. They were sampled in the spring of 2010 from Woburn Experimental Farm, Bedfordshire, UK. The soil at this site is a Cambric Arenosol (Avery and Catt, 1995). Soil 1 was under grassland and Soil 2 was from an adjacent long-term bare fallow plot (Table 1).

Six 50 g portions (dry weight equivalent) of each moist soil (sieved <2 mm) were pre-incubated at 40% water holding capacity (WHC) for 2 weeks in order to avoid artefacts caused by the initial flush of microbial activity after sieving. Since excess substrate is expected to be a requirement for significant EPS production (Nunan et al., 2003), the microbial biomass was stimulated by substrate addition. Glycerol was chosen as it yields no sugars or proteins on decomposition, and therefore, treatment induced differences in extracellular protein and carbohydrate could be used as reasonable indicators of substrate-induced EPS production. Six samples of each soil were thus brought to a water content of 50% WHC by the addition of either deionised water (control) or glycerol solution, to each of 3 replicates, delivering 2 mg C g^{-1} soil. Soil was incubated in glass cylinders at 25 °C for 10 days in the dark to prevent phototrophic inputs. Partially humidified air was passed through at a rate of 60 mL min^{-1} to induce the slow desiccating conditions previously linked to high EPS-polysaccharide production in sand (Roberson and Firestone, 1992).

2.2. Extraction techniques compared

Original and modified versions of both GRSP and WAEP extraction methods described below were compared. In both the original methods, the soil is required to be air dried before extraction. Due to the suspected hydraulic decoupling function described above we had hypothesised that an additional hydraulic coupling step (hydrating samples in the respective aqueous extractants prescribed in each method overnight) would increase the extraction efficiency of EPS, yielding more EPS from the pre-hydrated soils in comparison to the air dry soil. Each protocol is described below.

2.2.1. Weak acid extractable polysaccharide (WAEP) extraction

As per the protocol of Lowe (1994), 100 mL 0.5 M H_2SO_4 was added to 1 g air-dried soil and autoclaved for 60 min at 121 °C and 103 KPa. The extract was centrifuged at 5200 × *g* for 20 min (4 °C). Supernatant was transferred to 50 mL centrifuge tubes, and frozen in liquid N_2 before storage at −80 °C pending analysis. The modified extraction method (WAEPb) was similar to the WAEP protocol, except that soil was first hydrated with extractant the night preceding extraction. 1 g of each air-dry soil (aggregates <2 mm) was placed into 50 mL centrifuge tubes, with 20 mL 0.5 M H_2SO_4. Tubes were then shaken, packed in ice, at 2 cycles s^{-1} for 30 min, before transferring to 4 °C overnight. After 16 h, soil suspensions were transferred into 250 mL autoclavable flasks together with a further 80 mL of fresh 0.5 M H_2SO_4, then autoclaved and separated as above.

2.2.2. Easily extractable glomalin (EEG)/GRSP extraction

As per the protocol of Wright and Upadhyaya (1996), 20 mL of 20 mM citrate (pH 7) was added to 2.5 g soil, autoclaved for 30 min at 121 °C and 103 KPa, then centrifuged at 3200 × *g* for 20 min

Table 1
Soil properties.

Soil no.	Management	Organic C (%)	Total N (%)	C/N ratio	pH	Clay %	NO_3-N (μg g^{-1})
1	Bare fallow	0.299	0.029	10.3	5.53	7.9	0.01
2	Grassland	2.602	0.229	11.4	5.95	8.0	19.79

(4 °C). Supernatant was transferred to new 50 mL centrifuge tubes and frozen in liquid N_2 before storage at −80 °C pending analysis. In the modified EEG extraction protocol (EEGb), the procedure was as for the EEG protocol except that soil was first hydrated overnight with extractant: 2.5 g soil was placed into 50 mL centrifuge tubes and the citrate was added as before. Tubes were then packed in ice and shaken at a speed of 2 cycles s^{-1} for 30 min before transferring to 4 °C overnight. After 16 h the tubes were autoclaved and extracts prepared as per the original protocol.

2.2.3. Cation exchange resin (CER) extraction

EPS extraction buffer was prepared in 18 MΩ H_2O to: 2 mM $Na_3PO_4 \cdot 12H_2O$ (0.760 g L^{-1}), 4 mM $NaH_2PO_4 \cdot H_2O$ (0.552 g L^{-1}), 9 mM NaCl (0.526 g L^{-1}), 1 mM KCl (0.0746 g L^{-1}), then adjusted to pH 7 with 1 M HCl and cooled to 4 °C. The extractant for soluble microbial products (SMP) was prepared by adjusting 0.01 M $CaCl_2$ (local rainwater ionic equivalent) to pH 7 with 0.01 M $Ca(OH)_2$, and cooling to 4 °C.

In the original method used to extract EPS from activated sludge (Frolund et al., 1996), the quantity of CER required is calculated on the basis of Volatile Solid (VS) content in environmental sample. Sufficient CER, equal to 70 g DOWEX CER g^{-1} volatile solids (VS), was placed in another set of 50 mL centrifuge tubes, capped and cooled to 4 °C. The quantity of VS in the soil to be extracted was determined indirectly from a previous measure of C loss on ignition (LECO), thus CER (g) was calculated as:

$$((2.543 \times (\text{SOC\%} \times \text{soil sample mass (g DWE)})) \times 70\ \text{g})$$

where 2.543 is the conversion factor from LECO C% to VS, i.e. 4.63 g CER g^{-1} for Soil 1 (grassland), and 0.53 g CER g^{-1} for Soil 2 (fallow). Moist soil (2.5 g dry weight equivalent; at 35% of water holding capacity) from each microcosm was kept at 4 °C whilst weighing into 50 mL disposable centrifuge tubes (Greiner Ltd.).

Soluble microbial products (SMP) were extracted from soils by dispensing 25 mL pre-cooled $CaCl_2$ solution described above and shaking in a refrigerated end-to-end shaker (4 °C) at 2 cycles s^{-1} for 30 min. The soil/solution was then centrifuged at 3200 × *g* for 30 min, the SMP solution decanted into fresh tubes, frozen rapidly in liquid nitrogen and stored at −80 °C. To extract EPS, the pre-weighed CER was transferred to the remaining sample (centrifuge pellet) together with 25 mL chilled extraction buffer, shaken hard by hand to resuspend the pellet, and placed on the chilled shaker for a further 2 h. Samples were subsequently centrifuged at 4000 × *g* for 30 min and the supernatant transferred into new tubes using a plastic 10 mL pipette. The tubes of EPS extract were frozen in liquid N_2, and stored at −80 °C.

2.3. Characterisation of extracted EPS

Protein and humic fractions were estimated using a microplate adaptation of the Lowry assay (Redmile-Gordon et al., 2013). Total carbohydrate was measured using the phenol–sulphuric acid method of DuBois et al. (1956), and uronic acid using the method of Mojica et al. (2007). Each biological replicate was analysed in triplicate.

2.4. ATP content of soils and residues

Following EPS extraction, EPS depleted soil was washed by resuspending in 10 mL PBS (standard biological buffer), followed by centrifugation at 4000 × *g* for 10 min and discarding the supernatant. The pellet was quickly frozen at −80 °C pending ATP analysis. ATP was extracted from the centrifuge pellets alongside 2.5 g portions (DWE) of the experimental soils which had been frozen to −80 °C at the time EPS extractions commenced. Measurement of ATP was achieved using the method of Jenkinson and Oades (1979) as modified by Redmile-Gordon et al. (2011). An indicator of cell-lysis was provided by comparing microbial biomass ATP between soils and soil extract residues.

2.5. Statistical analyses

Student's *t*-tests (two tailed, equal variances) were applied using Genstat Software (VSN_International, 2011) to assess statistical significance of differences between means, of 1) soil microbial ATP concentrations in the soils, and residues following EPS extraction, and 2) EPS content between extracts of soils with and without additional substrate.

3. Results

3.1. Cell lysis

Microbial ATP, in soils provided with substrate before EPS extraction, amounted to 2.22 and 0.22 nmol ATP g^{-1} soil in the grassland and fallow soils, respectively. Microbial ATP remaining after each EPS extraction method is presented in Fig. 1. Both WAEP and EEG methods significantly decreased the measurable ATP ($p < 0.01$) to practically zero. Similar soil ATP concentrations before and after extraction with CER suggest that minimal disruption of cell membranes occurred (students *t*-test comparing ATP before with ATP after, $p = 0.86$ for arable, and $p = 0.75$ for fallow soil). ATP concentrations remaining after WAEPb and EEGb extractions (not shown) were no different from their unmodified counterparts (WAEP and EEG).

3.2. EPS extract comparison

Analyses of EPS extracts of fallow soil showed that among the compared methods, EEGb and CER were the only protocols to extract a statistically significant increase in EPS-polysaccharides due to substrate provision (Table 2; $p < 0.05$ for both protocols). Modification of both the WAEP and EEG protocols to include a hydraulic coupling step (WAEPb and EEGb) decreased the probability that the observed increases were simply a result of chance (Table 2). This modification also resulted in a bigger difference in uronic acid concentration between soils with and without substrate: about 6% and 16% more uronic acid was measured after

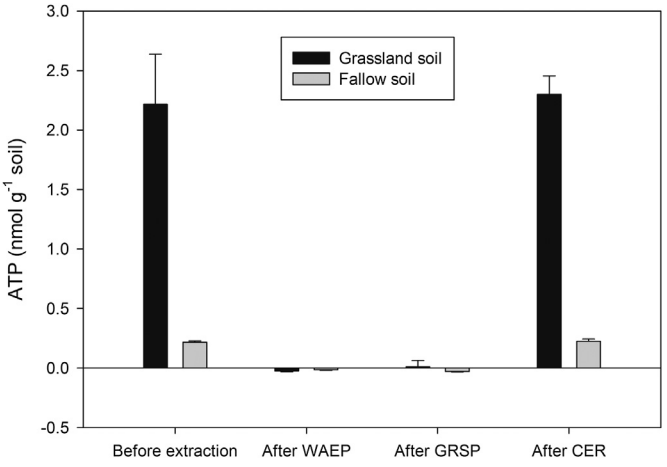

Fig. 1. Soil microbial ATP concentration before and after EPS extraction.

Table 2
EPS extract characteristics (Soil 1; fallow).

Content	Extract	Without substrate (μg g^{-1} soil)	±	With substrate (μg g^{-1} soil)	±	Difference (μg g^{-1} soil)	*p* value
Total polysaccharide							
	CER[a]	169	12	229	4	61	<0.01
	WAEP	3752	171	3919	128	167	0.48
	WAEPb	2760	89	3010	43	251	0.06
	EEG	926	16	986	22	60	0.09
	EEGb[a]	1141	12	1351	27	209	<0.01
Protein							
	CER	43	9	55	5	12	0.27
	WAEP	nd		nd		nd	
	WAEPb	nd		nd		nd	
	EEG	121	13	124	13	3	0.89
	EEGb	150	5	155	12	5	0.71
HAE							
	CER	136	22	163	6	28	0.28
	WAEP	nd		nd		nd	
	WAEPb	nd		nd		nd	
	EEG	1558	37	1548	68	−9	0.91
	EEGb[a]	2385	52	2638	33	253	0.02
Uronic acids							
	CER	65	5	78	0	12	0.06
	WAEP	1230	30	1236	11	5	0.87
	WAEPb[a]	443	8	471	4	28	0.04
	EEG	112	7	122	5	10	0.31
	EEGb[a]	168	8	195	4	27	0.04

nd indicates not detected.
[a] Indicates statistically significant difference due to addition of substrate with >95% confidence.

substrate was applied using WAEPb and EEGb, respectively. This was statistically significant when using modified protocols ($p < 0.05$), but not with the unmodified WAEP (Lowe, 1994) and EEG extractions (Wright and Upadhyaya, 1996).

A statistically significant increase in the humic acid equivalent (HAE) pool due to provision of substrate was also detected using the EEGb protocol. Both the EEG and EEGb protocols extracted between 10 and 20 times more HAE than did the CER extraction, but only 3 times more protein. The corresponding extract HAE/protein ratios were 3.16 and 2.96 obtained using CER (without and with substrate, respectively) contrasting with ratios of 15.90 and 17.02 when extracting with the EEGb protocol.

Table 3
EPS extract characteristics (Soil 2; grassland).

Content	Extract	Without substrate (μg g^{-1} soil)	±	With substrate (μg g^{-1} soil)	±	Difference (μg g^{-1} soil)	*p* value
Total polysaccharide							
	CER	401	39	461	9	60	0.21
	WAEP	15,633	529	16,010	371	377	0.59
	WAEPb	12,459	542	11,082	640	−1377	0.18
	EEG	6195	454	5479	108	−715	0.20
	EEGb	7278	425	7642	56	364	0.44
Protein							
	CER	163	9	177	7	14	0.30
	WAEP	nd	nd	nd	nd	nd	nd
	WAEPb	nd	nd	nd	nd	nd	nd
	EEG	1015	124	1269	19	254	0.11
	EEGb	1186	71	1154	19	−32	0.69
HAE							
	CER[a]	590	12	549	3	−41	0.03
	WAEP	nd	nd	nd	nd	nd	nd
	WAEPb	nd	nd	nd	nd	nd	nd
	EEG	16,199	592	17,455	962	1256	0.33
	EEGb	24,029	263	24,417	470	388	0.51
Uronic acids							
	CER	168	2	164	12	−4	0.76
	WAEP	1775	48	1712	52	−64	0.42
	WAEPb	885	37	906	15	21	0.64
	EEG	555	26	586	27	31	0.46
	EEGb	752	12	791	19	39	0.16

nd indicates not detected.
[a] Indicates statistically significant difference due to addition of substrate with >95% confidence.

With CER, approximately 3 times the total quantity of EPS-polysaccharide was extracted from Soil 2 (grassland control) than Soil 1 (fallow control; both without substrate addition). There was a similar absolute increase in extractable EPS-sugars with added substrate, although this increase was not statistically significant for the grassland soil (Table 3; $p = 0.21$). Indeed, no extraction protocol detected any statistically significant increases in EPS in response to substrate addition to Soil 2. However, the decrease in HAE extracted by CER after substrate addition was statistically significant.

4. Discussion

The conditions imposed in this study were expected to favour EPS production, previously observed as a survival response to desiccation (Roberson and Firestone, 1992). Analysis of CER and EEGb extracts both suggested that increased polysaccharide production by the soil microbial biomass had occurred. However, since only extracellular biopolymers can be classified as EPS, microbial ATP was used to indicate the proportion of cells not lysed during extraction. This study is not the first to use microbial ATP as an indicator of intracellular contamination. The ATP results of the present study are in accordance with Takahashi et al. (2009) suggesting that extraction with CER does not cause lysis of the microbial biomass, and therefore the statistically significant increase in detected EPS was unlikely to be an artefact of intracellular contamination (Table 2; $p < 0.01$).

The use of ATP as an indicator of lysis is questionable for the methods involving autoclaving steps (e.g. in both WAEP and GRSP methods) since some degree of non-enzymatic hydrolysis of ATP is also likely to occur at these high temperatures and pressures (Leibrock et al., 1995). Nonetheless, autoclaving alone is almost certain to cause extensive cell-lysis and contamination of extracts with intracellular material (Koga and Kusaka, 1968; Dabbagh et al., 1974; Shigehisa et al., 1991) and cause changes in the chemical structure of the extracted EPS (Sheng et al., 2010). In addition to specific concerns with autoclaving, more recently the use of H_2SO_4 and heating were both separately indicated to cause cell-lysis (Sun et al., 2012). With regard to EEG techniques, intracellular material was also found to be extractable by autoclaving (Driver et al., 2005). Our findings agree that neither method WAEP (Lowe, 1994) nor EEG (Wright and Upadhyaya, 1996) leave cells intact.

Even if intracellular contamination is accepted, using the WAEP protocol, no increase in polysaccharide concentration was detectable in either soil given substrate. Using the same technique, Tang et al. (2011) did not find an expected link between aggregate stability and polysaccharide concentration. In addition to intracellular microbial polysaccharides from autoclaving, heating soil with 0.5 M H_2SO_4 extracts significant amounts of residual plant polysaccharides, e.g. xylose and arabinose (Cheshire, 1979). It is not known what proportion of sugars were released through hydrolysis of glycosidic bonds, or through the dissolution of partially decomposed residues freed during rearrangement of supramolecular humic associations (see Piccolo, 2002) but the WAEP method resulted in approximately half of the total organic C being quantified as Dubois-reactive (3752 μg glucose equivalent g^{-1} soil; Table 2). For comparison, microbial biomass ATP in this soil before EPS extraction was very low (0.36 ± 0.11 nmol g^{-1} soil) which is approximately equivalent to a biomass C of about 30 μg g^{-1} of soil (Contin et al., 2001). Assuming the Dubois reactive fraction provides a reasonable estimate of sugars, more than 100 times the

quantity of biomass C would be estimated as EPS-polysaccharide. It seems highly unlikely that the majority of organic matter in the bulk fallow soil (no additional substrate) was actually EPS. Using imaging techniques, Chenu (1993) estimated that the total EPS (comprising mainly EPS-protein and EPS-polysaccharide) could contribute a maximum 80% of soil biofilm mass and typically not more than 1% of soil organic matter (Chenu, 1995). Nunan et al. (2003) also observed that a soil must be copiotrophic to facilitate substantial production of EPS in soil biofilms. The collated evidence concurs that the WAEP method is not a suitable approach to measure EPS.

The modified EEG method (EEGb) revealed a statistically significant increase in polysaccharide after provision of substrate to the fallow soil (Table 2). Total increases observed using modified WAEPb and EEGb methods were 251 and 209 μg g^{-1} soil, respectively, and of greater magnitude and statistical significance than indicated by the unmodified WAEP and EEG protocols (Table 2). The results from the present study therefore appear to support the hypothesis that desiccated biofilms require time to become hydrated before they can be successfully removed by aqueous extractants. Between the two hydraulically modified protocols, the increase in polysaccharide responding to substrate was only statistically significant when extracted from fallow soil using the EEGb method ($p < 0.01$; Table 2). In both the grassland and the fallow soils, extraction with CER yielded a similar absolute increase in EPS-polysaccharide afforded by provision of additional substrate (about 60 μg g^{-1} soil). However, the polysaccharide increases in grassland soil were not found to be statistically significant: not with any extraction method (Table 3). This lack of statistical certainty in the grassland soil was coupled with greater associated errors, probably arising from the large and heterogeneous background SOM content.

The acidic extractant used for WAEP and WAEPb methods prohibited colorimetric estimation of protein (Table 2). The GRSP content of EEG extracts is typically measured using the Bradford assay (Wright and Upadhyaya, 1996) however, protein content in EEG extracts was found to be more accurately quantified using a modified Lowry assay described by Redmile-Gordon et al. (2013). No statistically significant difference in EPS-protein content (responding to substrate) was found here in either soil, using any of the extraction methods tested. Poor recoveries of many proteins from soil are typical due to interference from clays or humic substances (e.g. Giagnoni et al., 2013). Furthermore the very low N availability in the fallow soil could have been a limiting factor for exudation of proteinaceous EPS.

Interestingly, measurements of EEGb extracts showed significantly more humic acid equivalents (HAE) from bare fallow soil when given substrate (253 μg HAE g^{-1} soil; $p = 0.02$). However, this apparent increase would be consistent with the theory that autoclaving in sodium citrate created humic artefacts from the increased biochemical pool as was discussed by Schmidt et al. (2011), e.g. via Maillard reactions occurring between amino acids and sugars (e.g. Evershed et al., 1997; Jokic et al., 2004). The observed increase in EEGb-extractable sugars alongside increases in EEGb-extractable HAE (Table 2) would seem to support this, and the autoclaving of soil in an aqueous extractant is not highly dissimilar to the initial procedures described for the synthesis of model 'humic acids' (e.g. Pompe et al., 1998). The estimation of this humic content is therefore suggested to be a useful indicator for (un)suitability of a method to extract EPS: indicating either the synthesis of artefacts, or the co-extraction of humified/pyrogenic soil organic matter.

Extractions using cool CER are not thought to induce humic artefacts (Sheng et al., 2010). Our results support this because the HAE/protein ratio obtained with CER extraction was lower than EEG techniques by more than an order of magnitude. Furthermore, the changes in EPS-polysaccharide concentration remained detectable. Curiously, a statistically significant difference in HAE was extracted from the grassland soil using CER (Table 3). Here, a decrease was observed due to substrate addition. Microbially mediated impacts upon decomposition rates of otherwise more chemically stable pools of SOM (or priming effects) are frequently affected by substrate additions and these are often increasingly positive with the degradability of substrate added (e.g. Pascault et al., 2013). The acquisition of C from more 'recalcitrant' SOM has been seen to account for a major part of the increases in microbial biomass observed after substrate addition (Bastida et al., 2013). Here, in the grassland soil, the decrease in CER-extractable HAE concentration ($p < 0.05$) supports previous observations that chemically more recalcitrant SOM can be affected by labile inputs, but the mechanisms are still not clearly understood and require deeper investigation.

Although there were statistically significant increases in uronic acid concentration observed due to substrate addition, these were detected only when using the modified extraction protocol WAEPb. Since glucuronic acid can be produced as a non-biological oxidation product of glucose (Fischer et al., 2007) oxidation by H_2SO_4 might explain the high uronic acid content obtained using both the WAEP and WAEPb techniques. It is not known exactly why WAEPb extracted less total uronic acid than WAEP – possibly the early addition of the acid for equilibration lowered the availability of H^+, and with it the propensity for oxidation during autoclaving, nonetheless, no real confidence can be taken from uronic acid concentrations given under these conditions.

Other existing methods to extract soil polysaccharide may warrant further investigation to determine the specificity for EPS. For example, methods involving hot water and H_2SO_4 such as compared by Puget et al. (1999) are likely to result in less intracellular contamination because they exclude the autoclaving step.

The principle behind EPS extraction with CER, is that a combination of shear forces and resin-Na^+ cause ion exchange with multivalent cations that link the EPS (primarily Ca^{2+} and Mg^{2+}) resulting in dissolution of the EPS macrostructure (Wilén et al., 2003; Sheng et al., 2010). This approach appears to be very effective at extracting extracellular proteins from the EPS matrix (D'Abzac et al., 2010). Our results concur with Liu and Fang (2002) and Simon et al. (2009) that CER extracts relatively large polysaccharide, protein and uronic acid fractions in comparison to indicators of cell lysis. However, it should be noted that the method proposed here should be expected to provide an underestimate of total soil EPS. For example, EPS bound by trivalent iron and aluminium may be less well represented by CER extraction in soil since the trivalent forms exchange less readily than divalent Mg^{2+} and Ca^{2+} (Wilén et al., 2003). Such bias was later observed in activated sludges (Park and Novak, 2007). More work is required to assess the inclusivity of CER extraction in soil.

In situations where iron-bound EPS is of specific interest, it might be suitable to develop a soils technique drawing upon the work of Nielsen and Keiding (1998) who used sulfide to reduce the Fe^{3+} linking anionic groups in EPS to insoluble FeS, thereby inducing disintegration of the EPS matrix. However, the operational exclusion of soluble microbial products (SMP) as described here (using a roughly isotonic extractant with salts of equal valence to the target EPS-binding site) is expected to be a crucial step preceding any EPS extraction from soil. The SMP exist as freely soluble extracellular polymers that are not bound to the cells (Comte et al., 2006a). The concentration of SMP in soil will be highly dependent upon hydrodynamics at the site shortly before sampling. This more ephemeral nature of SMP in relation to EPS, combined with the difficulty in chemically distinguishing between them, supports the approach for physical separation before extraction of EPS.

Physical agitation with CER is now the most popular extraction approach for EPS in active sludges (e.g. Ge et al., 2010). Takahashi et al. (2009) also recommend the use of CER for the extraction of EPS from benthic diatoms of intertidal sediments. The work conveyed here supports these related studies and represents a step towards more unified concepts within the environmental disciplines of soil, sediments and water. An inter-compatible approach will support use of complementary datasets and therefore help the construction of improved environmental models.

5. Conclusions

The statistically significant increases in EPS-polysaccharide measurements, validated by ATP, combined with the lowest estimate of HAE, identify the CER method described here as the most appropriate method currently available for estimating EPS content in soils.

In soils, the abbreviation 'EPS' has been used previously to refer to 'extracellular polysaccharides'. However, the bulk of literature from a variety of disciplines defines 'EPS' as being inclusive of numerous other components (protein, DNA, etc). In the interests of clarity for newcomers to the subject, and to foster cross-discipline relevance, we propose the term 'EPS' is used to describe the collective extracellular matrix, with component pools being qualified with an appropriate descriptor, e.g. 'EPS-protein' or 'EPS-polysaccharide'. Furthermore, when characterising biofilms in other disciplines, we hope researchers increase efforts to attempt distinction between EPS exuded by cells, and the exogenous decaying organic materials which are frequently co-extracted with EPS.

Understanding biofilm dynamics and their impacts in soils is expected to be a significant step towards understanding soil microarchitecture, and will require extensive further input from biological, chemical and physical disciplines.

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