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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.