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Untargeted soil metabolomics methods for analysis of extractable organic matter

Tami L. Swenson, Stefan Jenkins, Benjamin P. Bowen, Trent R. Northen*

Life Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA 94720, USA

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ABSTRACT

The cycling of soil organic matter (SOM) by microorganisms is a critical component of the global carbon cycle but remains poorly understood. There is an emerging view that much of SOM, and especially the dissolved fraction (DOM), is composed of small molecules of plant and microbial origin resulting from lysed cells and released metabolites. Unfortunately, little is known about the small molecule composition of soils and how these molecules are cycled (by microbes or plants or by adsorption to mineral surfaces). The water-extractable organic matter (WEOM) fraction is of particular interest given that this is presumably the most biologically-accessible component of SOM. Here we describe the development of a simple soil metabolomics workflow and a novel spike recovery approach using ^{13}C bacterial lysates to assess the types of metabolites remaining in the WEOM fraction. Soil samples were extracted with multiple mass spectrometry-compatible extraction buffers (water, 10 mM K_2SO_4 or NH_4HCO_3, 10–100% methanol or isopropanol/methanol/water [3:3:2 v/v/v]) with and without prior chloroform vapor fumigation. Profiling of derivatized extracts was performed using gas chromatography/mass spectrometry (GC/MS) with 55 metabolites identified by comparing fragmentation patterns and retention times with authentic standards. As expected, fumigation, which is thought to lyse microbial cells, significantly increased the range and abundance of metabolites relative to unfumigated samples. To assess the types of microbial metabolites from lysed bacterial cells that remain in the WEOM fraction, an extract was prepared from the soil bacterium *Pseudomonas stutzerii* RCH2 grown on ^{13}C acetate. This approach produced highly labeled metabolites that were easily discriminated from the endogenous soil metabolites. Comparing the composition of the fresh bacterial extract with what was recovered following a 15 min incubation with soil revealed that only 27% of the metabolites showed >50% recovery in the WEOM. Many, especially cations (polyamines) and anions, showed <10% recovery. These represent metabolites that may be inaccessible to microbes in this environment and would be most likely to accumulate as SOM presumably due to binding with minerals and negatively-charged clay particles. This study presents a simple untargeted metabolomics workflow for extractable organic matter and an approach to estimate microbial metabolite availability in soils. These methods can be used to further our understanding of SOM and DOM composition and examine the link between metabolic pathways and microbial communities to terrestrial carbon cycling.

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1. Introduction

1.1. Microbial products are an important source of soil organic matter

Over two-thirds of carbon in the terrestrial biosphere is stored as soil organic matter (SOM) originating from plant, animal and microbial sources (Johnston et al., 2004). Microorganisms are a critical component in soil as they are actively involved in the cycling of SOM (*i.e.* production of microbial products and metabolic

* Corresponding author. Tel.: +1 510 486 5240; fax: +1 510 486 4545.
E-mail address: TRNorthen@lbl.gov (T.R. Northen).

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decomposition) (Schmidt et al., 2012). The component of SOM that is accessible to microbial processing is the soluble fraction (Gregorich et al., 2000; Blagodatsky et al., 2010), referred to here as dissolved organic matter (DOM) and is physically defined as what can pass through a 0.45 μm filter. As with SOM, a significant portion of this complex pool of metabolites is thought to be derived from soil microbes with the flow of this carbon fraction mediating microbial biomass turnover (Baldock and Nelson, 2000; Gregorich et al., 2000; Kalbitz et al., 2000).

DOM is in a constant state of flux by soil microbes (Schimel and Schaeffer, 2012; Schmidt et al., 2012; Xu et al., 2014). However, microbial decomposition, and therefore the resultant composition of DOM, depends on the availability of small molecule substrates *in situ*. A number of biotic and abiotic factors affect microbial access to these molecules (such as leaching or diffusion), but one of the most critical factors is adsorption to mineral surfaces (Kalbitz et al., 2000). The types of biologically accessible substrates likely depend on the specific interactions between DOM components and mineral surfaces. In turn, the composition of the available substrate pool will be a major determinant of microbial community structure and metabolic activities (Judd et al., 2006). Hence, elucidation of the chemical composition of microbe-accessible substrates is a critical step toward understanding the complex dynamics of soil nutrients and microbial communities.

1.2. Traditional soil extraction methods and DOM analyses

To analyze DOM, a standard method is to obtain the water-extractable organic matter (WEOM) fraction by using aqueous extractants, while microbial biomass is measured by fumigating soil with chloroform vapors to release intracellular metabolites prior to extraction (Brookes et al., 1985; Vance et al., 1987). Comparisons are then made to an extraction of unfumigated soil in order to estimate the labile or microbe-accessible fraction. Given technical limitations on measuring its molecular composition, DOM has typically been quantified by oxidizing or combusting the soil sample and analyzing its elemental composition to determine dissolved organic (or inorganic) carbon or total dissolved nitrogen (Jones and Willett, 2006). In some cases, DOM and SOM are resolved into more specific classes of metabolites such as neutral sugars, amino sugars, amino acids, fatty acids and other biomolecules using colorimetric methods or compound-specific derivatization followed by gas chromatography/mass spectrometry (GC/MS) (Amelung et al., 2008; Kakumanu et al., 2013). Such groupings into broad classes of biomolecules essentially preclude linkage with microbial genomics since biochemical specificity requires knowledge of molecular composition.

1.3. The use of untargeted metabolomics to understand DOM composition and availability

Untargeted metabolomics is a rapidly-growing and robust method that has become an important approach in biomedical science by providing comprehensive data-driven metabolism analyses of complex extracts (Fiehn, 2002; Garcia et al., 2008; Baran et al., 2009, 2013). As mass spectrometry (MS) instruments such as Fourier Transform Ion Cyclotron Resonance (FTICR/MS) (Hirai et al., 2004), capillary electrophoresis (CE/MS) (Soga et al., 2003; Edwards et al., 2006), and liquid chromatography (LC/MS) (Baran et al., 2011, 2013) are commonly used, they are not widely available. In contrast, GC/MS is a widely-used and generally more-accessible instrument to microbiologists and soil scientists due its low operational cost, availability of curated metabolite spectral databases, broad analytical scope with good coverage of metabolite classes (carbohydrates, alcohols, sterols, amino acids, fatty acids, *etc*), as well as widespread application to phospholipid fatty acid analysis (Buyer and Sasser, 2012). It would therefore be desirable to develop workflows for GC/MS-based soil metabolomics for examination of extractable organic matter composition (Fiehn et al., 2000; Roessner et al., 2000; Koek et al., 2006; Lee et al., 2012).

Metabolomics approaches are beginning to have a major impact on improving our understanding of marine and freshwater communities and the significant role DOM plays in these environments. While the majority of marine metabolomics studies have involved controlled cultures, metabolite profiling has been successful with marine bacteria, microalgae, macroalgae and animals (Mopper et al., 2007; Minor et al., 2014). Metabolomics analyses of spent media from these systems have shed light on unique chemical defense mechanisms and production of secondary metabolites (Goulitquer et al., 2012). Marine metabolomics has also contributed to our understanding of DOM lability and recalcitrance as a function of microbial carbon pumps in aquatic systems (Jiao et al., 2010). Studies such as these are allowing us to grasp the value of DOM in shaping microbial communities and to begin to extrapolate its role in global climate change. Unfortunately, the field of soil metabolomics (and our understanding of SOM) lags behind, but is emerging as an equally important area of study.

1.4. Soils present many challenges to untargeted metabolomics methods

Recently a few papers have emerged relating to metabolomics of soils, many pertaining to the production of osmolytes during drying and re-wetting conditions (Kakumanu et al., 2013; Warren, 2013, 2014; Jones et al., 2014). FTICR/MS is one of the most established methods for DOM analysis and routinely resolves thousands of unique chemical formulas. However, these methods are typically used to discriminate soils and soil treatments based on changes in abundance and composition of these chemical formulas (Hockaday et al., 2006; Ohno et al., 2014). Understanding the soil biochemistry requires identifying the soil metabolites such that they can be linked to enzymes and microorganisms. Towards this goal, a recent pioneering study by Warren describes important methods for identifying and quantifying soil metabolites using CE/MS and GC/MS (Warren, 2014). Findings from this study provide key insights into the drying response of soil bacteria in terms of osmolyte production and accumulation.

The comparatively sparse literature on metabolomics of soils versus marine systems is likely attributable to the unique challenges soils present for untargeted metabolomics. To evaluate the chemical composition of SOM or DOM, methods typically require the development of analytical approaches similar to the classical soil extraction methods involving chloroform fumigation followed by extraction with buffers containing high concentrations of salts (*e.g.* 500 mM K_2SO_4) (Brookes et al., 1985; Vance et al., 1987; Tate et al., 1988; Murage and Voroney, 2007; Makarov et al., 2013). Unfortunately the salt content of the resultant samples complicate metabolite analysis because of salt crystal formation during dry-down and ion suppression during electrospray ionization mass spectrometry analysis (Annesley, 2003). To overcome these challenges and setbacks, optimizing detection of a large range of soil metabolites by GC/MS requires careful consideration of numerous variables during method development including extractant selection, extraction time, sample concentration and derivatization methods.

1.5. Untargeted global metabolite profiling with GC/MS

As is evident, classical soil extraction methods involve long extraction times followed by compound-specific analyses. However,