A Method for Determining Community Level Physiological Profiles of Organic Soil Horizons

Community level physiological profiles (CLPPs) have been widely used to assess microbial community diversity in soils. Refinement of techniques to determine soil CLPP eventually led to the development of the MicroResp technique. This technique avoids many of the pitfalls of earlier methods by using a whole-soil approach coupled with the convenience of microplates. However, issues related to soil pretreatment (primarily sieving) can arise when using the standard MicroResp method to determine the CLPPs of forest floors. Here, we developed a modified multiple substrate induced respiration (multi-SIR) method that lessens the effects of pretreatment by using a larger soil volume in custom 24-well deep-well plates. Microbial community indices including catabolic evenness ($E$) and CLPP were determined on a range of forest soils using both the standard MicroResp and our modified multi-SIR method. The modified method reduced the variation among the triplicate substrate wells and displayed a wider range of $E$ among the soils measured. Additionally, using multivariate nonmetric multidimensional scaling (NMDS) as well as cluster analysis, we found that the modified method was able to better detect differences in soil CLPPs. The standard MicroResp method remains a valuable technique for many soils; however, our modified multi-SIR method is more suitable for organic soils, such as forest floors, that have low bulk density.

Abbreviations: CLPP, community level physiological profiles; $E$, catabolic evenness; multi-SIR, multiple substrate induced respiration; NMDS, nonmetric multidimensional scaling; UV-Vis, ultraviolet-visible.

Microbial community diversity is an effective measure to illustrate the effects of natural and man-made disturbances on soil (Griffiths and Philippot, 2013). Strategies to measure community diversity often employ biochemical, genetic, or physiological indices (Hill et al., 2000). Biochemical and genetic profiling techniques include broad-scale phospholipid fatty acid analysis, molecular sequencing of 16S ribosomal ribonucleic acid (rRNA) genes, or more recent metagenomic deoxyribonucleic acid (DNA) analysis. While these allow characterization of the structural diversity of soil microbial communities, physiological indices are a better indication of essential soil functions such as biogeochemical cycling (Singh et al., 2014). Characterization of soil functional diversity may target analysis of individual enzymes or substrate-induced respiration measurements in the presence of added organic substrates (Degens and Harris, 1997). In particular, CLPPs that assess microbial catabolic diversity are very well suited to follow microbial functional adaptation to environmental stress and to test fundamental questions linked to soil biological resistance and resilience.
Community level physiological profiles were first determined using the BIOLOG method (Garland and Mills, 1991). However, as highlighted by Degens and Harris (1997) BIOLOG relies on nonsoil incubation conditions. Additionally, BIOLOG results may not provide an accurate estimation of the entire soil microbial community as inoculum preparation, substrate selection, and length of incubation can favor the expression of only a subset of the original community (Konopka et al., 1998). The potential issues with BIOLOG led to the development of the multi-SIR approach, which uses a whole-soil approach (Degens and Harris, 1997). While effective, multi-SIR was complicated and time consuming. To increase sample throughput, Campbell et al. (2003) developed the MicroResp method, which is based on the whole-soil approach of multi-SIR but benefits from the efficiency of rapid sample analysis provided by microplates. Since its development, the MicroResp method has been found to discriminate soil microbial community differences that may not be detected by multi-SIR (Chapman et al., 2007).

The MicroResp method has been extensively used in peatlands (Curlevski et al., 2011) as well as in a variety of forested settings including undisturbed mineral soils (Gömöryová et al., 2013) and forest litter layers (McIntosh et al., 2013), soils after fire (Ginzburg and Steinberger, 2012), and in restored post-mining landscapes (Banning et al., 2012). However, working with organic forest layers can present a challenge, as they can have low bulk density (Redding et al., 2005) compared to mineral soils making it difficult to ensure an adequate representative sample in small analysis volumes. The standard MicroResp soil pretreatment requires that soils be sieved to <2 mm to ensure that soil samples freely fill the wells. Using only the <2-mm fraction may miss a large portion of the forest floor, as Fogel and Hunt (1979) demonstrated that up to two-thirds of sieved forest floor are retained in size fractions ranging from 2 to 25 mm. Additionally, sieving to 2 mm compared to 5 mm can cause increased metabolism rates of low molecular weight organic compounds when applied to deciduous and coniferous forest floors (Datta et al., 2014). Finally, using a coarser sized sieve could address these issues but would also introduce new problems with sample loading as the size of coarser sieved organic materials would be similar or greater than the diameter of the microwells used in the MicroResp method.

The following study was conducted in an attempt to solve the issues encountered when using the MicroResp method with organic samples of low bulk density and size fractions >2 mm, such as forest floor layers. Here, we determined CLPP for forest soils with the same process as the MicroResp method but with larger soil volumes and without sieving. We used a modified 24-well microplate design instead of the 96-well microplates used in the standard MicroResp analysis. We hypothesized that the larger, modified 24-well apparatus would decrease the variability among replicate samples, hence, increasing the precision of the method. We further hypothesized that the power of the 24-well approach to distinguish among soil types would be greater than that of the traditional 96-well method.

**MATERIALS AND METHODS**

**Modification of the MicroResp Method Custom Apparatus**

Many components for the 24-well apparatus had to be custom built, as commercially available components did not exist (Fig. 1). Custom built 24-well deep-well blocks and silicon gaskets for substrate loading were manufactured by a local supplier (Gemma Plastic Products Inc., Edmonton, AB). The deep-well blocks were created from a 12.5- (length) by 8.5- (width) by 5.0- cm (height) block of solid acrylic with machine lathed deep wells. Each well was 3.5 cm deep with well spacings and diameters identical to commercially available 24-well microplates. Gaskets were constructed from mold injected silicon rubber and made to fit a 24-well microplate. The base of the gaskets was 12.5 (length) by 8.5 (width) by 0.4 cm (height). Each gasket had raised cylindrical extensions roughly 3 mm thick on either side of the gasket base to ensure an adequate seal between the agar detection plate and the deep-well block. A perforation was located at the center of each extension to allow gas flow through the gasket. A custom filling device was manufactured in the lab (Swallow) using a 24-well microplate with the well bottoms removed along with a housing made of three acrylic sheets glued to the sides of the modified microplate.

**Detection Agar**

The colorimetric detection agar was prepared as instructed in the MicroResp technical manual (Cameron, 2007) by combining a
solution of 3% purified agar (Oxoid) with indicator dye containing cresol red (18.75 mg L$^{-1}$), KCl (16.77 g L$^{-1}$), and sodium bicarbonate (0.315 g L$^{-1}$). Detection wells in 96-well microtiter plates received 0.15 mL of colorimetric detection agar while detection wells in 24-well microtiter plates received 0.5 mL. Upon preparation, all detection plates were covered with parafilm and left to equilibrate for 1 wk in a sealed chamber containing soda lime and a beaker of deionized water.

**Calibration Curve and Detection Plate Correction**

Calibrating the ultraviolet-visible (UV-Vis) absorbance of the colorimetric detection agar at varying concentrations of CO$_2$ was done using a plate reader and a HP 5890 gas chromatograph equipped with a 1-m Porapak Q column and an Agilent GC sample 80 autosampler. Absorbance (572 nm) and CO$_2$ concentrations were determined from incubation microcosms constructed using 130-mL flat bottomed test tubes (Pyrex brand) and Suba-Seal rubber septa. Each microcosm contained an 8-well strip of a 96-well strip plate (covered in cheese cloth to prevent soil particle contamination) and one sample of soil as described in Table 1. Varying concentrations of CO$_2$ were generated by incubating the sealed microcosms for different lengths of time (between 2 and 5 h). After incubation, CO$_2$ was collected from the headspace of the sealed microcosms using a gas-tight syringe and stored in evacuated vials until measurement on the gas chromatograph. Once gas sampling was complete, the microcosms were opened and the 8-well strips were read for UV-Vis absorbance. The data were curve fitted using the function finder based on the bioscience family of equations from a curve-fitting website (Phillips, 2014). The final hyperbolic with offset relationship (Fig. 2) was selected out of a total of 78 equations, as it had one of the lowest sum of squared absolute error from the fitting procedure and the fourth highest $r^2$ value (the three equations with higher $r^2$ only improved $r^2$ at the fourth decimal and contained more equation variables than the relationship we selected).

An additional correction factor for the calibration curve had to be generated for the 24-well plates, as these had a faster CO$_2$ absorbance rate than the 96-well plates due to their larger surface area and shallower agar depth. Individual 96-well and 24-well agar detection plates were placed together in desiccators containing a beaker of distilled water along with either soda lime or soil and incubated for different periods of time to allow for different levels of CO$_2$ absorption. After incubation UV-Vis absorbance results were averaged for all wells on a given detection plate, the relationship between the 96-well and 24-well absorbance values was described using a linear regression based on the plate-average (Fig. 3). The intercept and slope of the regression line was then applied to any future 24-well absorbance values so that the absorbance readings could be quantified as percentage of CO$_2$ on the calibration curve.

**Community Level Physiological Profiles**

**Soil Characteristics and Sample Collection**

The soil types used were a combination of archived (air-dried) samples and recently collected soil samples from reclaimed and natural sites in Northern Alberta, Canada (Table 1. Description of soils used in the 24-well and 96-well MicroResp comparison.)

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**Table 1. Description of soils used in the 24-well and 96-well MicroResp comparison.**

<table>
<thead>
<tr>
<th>Soil type</th>
<th>Site type</th>
<th>Forest type</th>
<th>Soil horizon</th>
<th>Treatment/storage</th>
<th>Collection year</th>
<th>Moisture Content</th>
<th>Soil per 96-well</th>
<th>Soil per 24-well</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Natural</td>
<td>Aspen</td>
<td>Organic (FH)</td>
<td>Sieved (4 mm) &amp; air dried</td>
<td>2008</td>
<td>284</td>
<td>0.04</td>
<td>0.31</td>
</tr>
<tr>
<td>B</td>
<td>Natural</td>
<td>Spruce</td>
<td>Organic (FH)</td>
<td>Sieved (4 mm) &amp; air dried</td>
<td>2008</td>
<td>308</td>
<td>0.05</td>
<td>0.38</td>
</tr>
<tr>
<td>C</td>
<td>Natural</td>
<td>Aspen</td>
<td>Mineral (0–10 cm)</td>
<td>Refrigerated (4°C)</td>
<td>2013</td>
<td>12</td>
<td>0.33</td>
<td>2.78</td>
</tr>
<tr>
<td>D</td>
<td>Reclaimed</td>
<td>Peat/mineral mix</td>
<td>Mineral (0–10 cm)</td>
<td>Sieved (4 mm) &amp; air dried</td>
<td>2009</td>
<td>28</td>
<td>0.26</td>
<td>2.44</td>
</tr>
<tr>
<td>E</td>
<td>Reclaimed</td>
<td>Forest floor/mineral mix</td>
<td>Mineral (0–10 cm)</td>
<td>Sieved (4 mm) &amp; air dried</td>
<td>2009</td>
<td>78</td>
<td>0.15</td>
<td>1.13</td>
</tr>
<tr>
<td>F</td>
<td>Natural</td>
<td>Aspen/spruce</td>
<td>Organic (FH)</td>
<td>Refrigerated (4°C)</td>
<td>2013</td>
<td>194</td>
<td>0.04</td>
<td>0.43</td>
</tr>
<tr>
<td>G</td>
<td>Natural</td>
<td>Aspen/jack pine</td>
<td>Organic (FH)</td>
<td>Refrigerated (4°C)</td>
<td>2013</td>
<td>119</td>
<td>0.09</td>
<td>0.74</td>
</tr>
<tr>
<td>H</td>
<td>Natural</td>
<td>Aspen</td>
<td>Organic (FH)</td>
<td>Refrigerated (4°C)</td>
<td>2013</td>
<td>91</td>
<td>0.05</td>
<td>0.46</td>
</tr>
</tbody>
</table>

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![Fig. 2. Fitted relationship between colorimetric detection agar absorbance (572 nm) and headspace percentage of CO$_2$ measured in the incubation vials using 8-well agar microstrips.](image1.png)

![Fig. 3. Fitted relationship between absorbance (572 nm) of colorimetric detection agar contained in 96-well and 24-well microplates under different concentrations of CO$_2$.](image2.png)
1). Upland forests in the region typically contain pure and mixedwood canopies of aspen (Populus tremuloides Michx.), white spruce (Picea glauca (Moench) Voss), and jack pine (Pinus banksiana Lamb.). The soils were chosen on the basis of ensuring a wide range of community function derived from their different origins and storage conditions. While not recommended when measuring microbial community function, some of the analyses were performed on air-dried samples to warrant strong structural and compositional differences when compared to the fresh samples. The comparison of samples with strong function differences was then used to test the detection abilities of the 96-well and 24-well methods. Further, this allowed us to investigate how these abilities were potentially affected by the different sample preparation procedures as well as the different sample amounts used by the two methods.

The archived air-dried soils were rewetted using a modified soak and drain method (Puustjäärvi, 1973), where soils were placed on a 0.5-mm sieve, saturated overnight in distilled water, and then drained of all gravimetric water. Field fresh soils were stored in a refrigerator for approximately 1 mo before analysis. Soils were transferred to aluminum trays, weighed, covered, and incubated for 1 mo at 23°C to allow suitable equilibrium of the microbial communities. Soil moisture was maintained by weighing the samples weekly and rewetting to their original soil weights with distilled water.

**Substrates**

The substrates were adapted from the MicroResp technical manual (Cameron, 2007), and were partly chosen based on availability in the laboratory. Stock solutions for 15 substrates were prepared using distilled water and further diluted accordingly for each soil based on the soil moisture content. Aliquot concentrations were specific to each soil so that the final substrate concentration in the soil solution would be 30 mg g⁻¹ soil-water for substrates with higher solubility (L-arabinose, L-lysine, citric acid, gluconic acid, L-malic acid, D-fructose, D-galactose, oxalic acid, succinic acid, N-acetyl glucosamine). For substrates with lower solubility (L-alanine, L-arginine, L-cysteine, L-histidine, oxalic acid, succinic acid, N-acetyl glucosamine).

Substrates were loaded on commercially available 96-well deep-well plates in 25-μL aliquots while the 24-well deep-well blocks received substrates in 250-μL aliquots before analysis. Each substrate was loaded into three replicate wells for a total of 48 wells per soil sample when distilled water was included. The smaller 96-well plates allowed for the analysis of two soil samples per deep-well plate, while the 24-well plates required two deep-well plates per soil sample.

**Incubation and Experimental Design**

Soils were analyzed using the conventional MicroResp method (96-well) and the 24-well multi-SIR method on the same day. Soils analyzed with the 96-well method had to be sieved to 2 mm so that a consistent amount of material would be added to each well while soils analyzed with the 24-well method were sorted and had only coarse roots, wood, and stones (when present) removed. Sieved soils were loaded onto the conventional MicroResp filling device while sorted samples were loaded on a custom built 24-well filling device. Before the addition of soil, the filling devices were securely mounted on top of the prepared deep-well plates and blocks.

Colorimetric detection plates were read for preincubation absorbance at 572 nm and then attached to deep-well plates immediately after the deep-well plates were loaded with soil. The 96-well detection plates were attached using the MicroResp gasket while 24-well detection plates were attached with the custom made gasket. Samples were incubated at 30°C in a chamber that contained an oscillating fan to minimize any internal temperature fluctuation. The 96-well samples were incubated for 6 h as suggested in the MicroResp technical manual (Cameron, 2007). The 24-well samples were incubated for 1.5 h, as on initial testing we found that incubation times greater than 3 h produced saturated colorimetric detection plates. After incubation, detection plates were removed from their deep-well counterparts and reread at 572 nm. The final CO₂ production rates (micrograms CO₂–C g⁻¹ dry soil h⁻¹) were determined by reading the equivalent percentage of CO₂ from the calibration curve (after correcting the 24-well values) while factoring in the headspace of the 96-well and 24-well deep-well plates, the soil dry weight, and the incubation time and temperature.

**Data and Statistical Analyses**

All statistical analyses were conducted using R (R Core Team, 2014). Variability in the amount of soil delivered by the filling device was tested by determining the coefficients of variation for each substrate used in the 96-well MicroResp and 24-well multi-SIR methods. Coefficients of variation for all colorimetric detection plate results were determined after dividing the postincubation absorbance of the three substrate wells by their preincubation readings (n = 288).

Soil substrate use values were based on averaging the triplicates wells for each of the 15 substrates. Ability of the 96-well and 24-well methods to differentiate soil communities based on substrate use was determined by calculating E as explained in Degens et al. (2001). Coefficients of variation and E data were analyzed using a one-way ANOVA after testing for parametric statistical assumptions.

Multivariate NMDS provided by the Vegan package (Oksanen et al., 2014) was used on all substrates to compares CLPPs of the soils. The NMDS used Bray–Curtis dissimilarities based on substrate CO₂ production rates that were Wisconsin double standardized after being square root transformed. Similarities in CLPPs among soils in both 24-well and 96-well methods were determined using a cluster analysis from the cluster package (Maechler et al., 2014). The cluster analysis used a Bray–Curtis measure of dissimilarity and a flexible β linkage method with a β value of ~0.25. Optimal leaf pruning of the cluster tree was done using indicator species analysis from the labdsv package (Roberts, 2013) where the tree which resulted in
the highest number of relevant indicator species ($p < 0.05$) was selected (McCune and Grace, 2002).

RESULTS AND DISCUSSION

As shown on Fig. 4a, the 96-well and 24-well methods produced coefficients of variation less than 10% across substrate responses. However, the mean coefficient of variation in the 24-well method was consistently lower ($p = 0.002$). Additionally, the 24-well method standard error (0.28) was half as large as the 96-well method standard error (0.46). Mean $E$ (Fig. 4b) was also lower in the 24-well method ($p < 0.001$), but the standard error was larger for the 24-well (0.67) than for the 96-well method (0.22).

The NMDS produced a two-dimensional ordination with a stress of 8.89 after two convergent solutions were found in six iterations. Soil samples analyzed with the 24-well method showed distinct spacing within the ordination space (Fig. 5a). In contrast, soil samples analyzed using the 96-well method were closely grouped with much less spacing within the ordination space. Cluster analysis after branch pruning showed that the 96-well and 24-well samples split into two separate groups (Fig. 5b). Soils analyzed with the 24-well method further divided into two even groups, while all soils analyzed with the 96-well method stayed grouped together.

The triplicate well measurements produced robust respiration results for the range of substrates used by the 96-well and 24-well multi-SIR methods, and both methods had low coefficients of variation. The 24-well method provided a slightly more precise measurement that may be attributed to the larger quantity of soil used with that apparatus. Compared to the 96-well method, the 24-well method displayed a wider range of $E$ across soil types. Results from the 24-well analyses displayed a standard error three times larger than those of the 96-well analyses, indicating a wider spread of the data.

The sieving pretreatment may have caused a more homogenous measure of $E$ in the 96-well method, as visual inspection after sieving showed large amounts of partially decomposed litter, coarse roots, and charcoal fragments >2 mm being retained on the sieves. In contrast, while the 24-well method also excluded coarse roots from analysis, a larger portion of litter was retained compared to the 96-well method. Sieving to <2 mm may have caused a strong homogenizing effect, which weakened the ability of the 96-well method to distinguish differences in functional diversity. In contrast, the 24-well method was able to retain distinct litter components and better captured the heterogeneity of each sample.

It should be noted that the scope of this study was limited to comparing the effectiveness of the 24-well and 96-well methods to detect differences among microbial communities and did not contain sufficient replication to study categorical effects on community diversity. However, there were a number of interesting community observations elucidated by the NMDS and cluster analysis, which can be addressed in relation to the known literature. A broad examination of the NMDS shows that the 24-well method was able to better distinguish differences in CLPPs among the different soils as the 96-well samples grouped much more closely in ordination space.

When focusing on the 24-well method, each type of fresh forest floor (Soils F, G, and H) occupied unique regions of the ordination space. This is a well-documented phenomenon, where the forest canopy type can be used as a descriptor to categorize and compare microbial community composition (Grayston and Prescott, 2005; Hannam et al., 2006; Ushio et al., 2008).
cation of Soils A and B in ordination space away from the other 24-well soils (particularly the fresh aspen) is most likely related to changes in microbial communities caused by long-term storage and rewetting after air drying (Zelles et al., 1991; Fierer et al., 2003). Interestingly, soils with mineral components (Soils C, D, and E) were grouped in similar ordination space and included Soil G, which on visual inspection, contained a large mineral soil component inadvertently intermixed with the forest floor during sample collection. Although the exact proportion of mineral component was not quantified, other forest floor samples did not contain visible traces of intermixed mineral soil. Boreal forest floors and their underlying mineral soils have distinct microbial communities (Hynes and Germida, 2013), and the addition of mineral soil may have altered the community response to the extent that the community diversity of Soil G was similar to the mineral soil samples (Soils C, D, and E). Soils C, D, E, and G were also identified by the cluster analysis as being distinct and clustered in Group 2, while all soils for the 96-well method and forest floor samples from the 24-well method were contained within Group 1.

The lack of CLPP distinction among samples analyzed using the 96-well method may again be due to the removal of coarser forest floor components by the sieving pretreatment, as it has been found to influence microbial diversity in mineral soils (Thomson et al., 2010). This effect may be more pronounced in organic layers of the forest floor, as they are home to microbial communities that differ vertically within a few centimeters in response to different stages of organic matter decomposition (Leckie et al., 2004). Retention of these coarser components may be necessary in determining overall microbial community diversity, which can drastically change as leaf litter undergoes decomposition (Aneja et al., 2006) and can be influenced by other forest floor components such as char (Pietikäinen et al., 2000). The larger soil quantity used in the 24-well method increases the forest floor components such as char (Pietikäinen et al., 2000). Composition (Aneja et al., 2006) and can be influenced by other diversity, which can drastically change as leaf litter undergoes decomposition (Thomson et al., 2010). This effect may be more pronounced has been found to influence microbial diversity in mineral soils (Soils A and B) in ordination space away from the other 24-well soils (particularly the fresh aspen) is most likely related to changes in microbial communities caused by long-term storage and rewetting after air drying (Zelles et al., 1991; Fierer et al., 2003). Interestingly, soils with mineral components (Soils C, D, and E) were grouped in similar ordination space and included Soil G, which on visual inspection, contained a large mineral soil component inadvertently intermixed with the forest floor during sample collection. Although the exact proportion of mineral component was not quantified, other forest floor samples did not contain visible traces of intermixed mineral soil. Boreal forest floors and their underlying mineral soils have distinct microbial communities (Hynes and Germida, 2013), and the addition of mineral soil may have altered the community response to the extent that the community diversity of Soil G was similar to the mineral soil samples (Soils C, D, and E). Soils C, D, E, and G were also identified by the cluster analysis as being distinct and clustered in Group 2, while all soils for the 96-well method and forest floor samples from the 24-well method were contained within Group 1.

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