

Optimization of an oxygen-based approach for community-level physiological profiling of soils

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ABSTRACT

Current approaches for rapid assessment of carbon source utilization by whole soil communities (i.e., community-level physiological profiling or CLPP) provide a limited, biased view of microbial communities with little connection to *in situ* activities. We developed an alternative CLPP approach based upon fluorometric detection of dissolved oxygen consumption in a microtiter platform which offers flexible manipulation of experimental factors. In the attempt to reduce oxygen re-dissolution, the wells were filled with liquid to very near the top and sealed. We found that filling the wells with 240 vs. 150 μl of sample improved the sensitivity of the system to discern both the response to a substrate amendment as low as 10 mg l^{-1} and un-amended, endogenous respiration. The preparation of a soil slurry facilitates inoculation into the microplate. Disruption of soil samples had a limited effect on the endogenous respiration in comparison to intact soil microbags in a 24-well microplate. Storage time (up to 33 days) reduced the level of activity in intact soil microbags but not in disrupted samples. A microcosm fertilization experiment was set to study the effects of N availability on the respiratory response in the plates. The use of soil organic carbon (SOC) and amended C-substrates (50 mg l^{-1}) was increased by the addition of nitrogen (N) in the plate, and appeared N-limited shortly after microcosm fertilization. The addition of the eukaryotic inhibitor cycloheximide delayed the initial increase in fluorescence (time to minimum response) of several C sources (casein, acetate, asparagine, coumaric acid), varying among soils, which could be explained by the fungal use of these compounds. However, the extent of the inhibition caused by cycloheximide did not increase at higher fungal to bacteria ratios as estimated by PLFA analysis, indicating that the direct estimation of the fungal biomass from cycloheximide addition is not feasible. This paper provides an optimized, standardized protocol for soil analysis, and sets the basis for further validation studies that will continue to define the underlying capabilities/biases of this approach.

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1. Introduction

Rapid, multivariate profiling of microbial communities based on physiological traits (i.e., community-level physiological profiling or CLPP) was first developed by Garland and Mills (1991) using Biolog microplates to compare different communities of heterotrophic soil bacteria. Since then, it has been used for the past two decades to study microbial functional diversity in a variety of terrestrial habitats, including drilling mud and surface cores (Lehman et al., 1995), compost (Insam et al., 1996), and agricultural and forest soils under different land-uses (Busse et al., 2001; Gomez et al., 2004; Winding and Hendriksen, 2007). While effective for simple, easy characterization of soil microbial communities, significant issues

limit the method's usefulness. Several authors have extensively reported the advantages and disadvantages of the Biolog method (Winding and Hendriksen, 1997; Preston-Mafham et al., 2002; Garland et al., 2007; Chapman et al., 2007). The method involves measurement of microbial growth in microtiter plates 24–72 h after inoculation, thus being a culture-dependant technique. The requirement for growth under high substrate concentrations (i.e., > 100 mM) causes severe selective enrichment, resulting in evaluation of only a small fraction of the overall community (Smalla et al., 1998; Ros et al., 2008). The defined, proprietary ingredients in the Biolog plates lead to inflexible and limited incubation conditions, which preclude testing of nutrient effects on carbon source utilization. The redox dye necessitates a buffered pH of 7.2 and also limits measurement of fungal activity (Preston-Mafham et al., 2002).

It is also possible to obtain a catabolic fingerprint of the microbial community by measuring the amount of CO_2 respired

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after the addition of a wide range of substrates, i.e., a multiple substrate-induced respiration (SIR) approach (Degens and Harris, 1997; Campbell et al., 2003). Degens and Harris (1997) first applied the multiple-SIR technique to measure the catabolic diversity of whole soil microbial communities. Their method consists of short-term incubations (4 h) of slurried soil in glass jars amended with different substrates in concentrations ranging from 10 (amino acids) to 100 mM (carboxylic acids) and subsequent headspace gas analysis. Although this method allows the measurement of indigenous microbial activity before growth of microorganisms occurs, its drawbacks are many: it is labor-intensive and requires high concentrations of substrates (Garland et al., 2007). To overcome some of these constraints, Campbell et al. (2003) developed a microrespirometry method that can be performed in a deep 96-well microplate, which has the advantage of being logistically feasible, suitable for running many replicates/samples at once, and adaptable to reading in existing plate readers (i.e., automated measurement of evolved CO₂). One major advantage of the MicroResp™ method is its flexibility, given the option to use different detection systems (Chapman et al., 2007). One detection method relies on a colorimetric reaction after trapping of CO₂ in alkali, which is set in a gel with a pH indicator dye. The other method makes use of radioactive substrates to detect ¹⁴C trapped in alkali-soaked filter paper and subsequent radioactivity measurement in a microplate liquid scintillation counter. Although it allows testing of low substrate concentrations, the cost and feasibility of using ¹⁴C-labeled compounds limit the adoption of this approach. As for the colorimetric assay, the concentrations of substrates necessary to detect gas in the headspace are as high as in the non-automated method by Degens and Harris (1997).

Quantifying dissolved O₂ consumption instead of CO₂ evolution may allow for improved sensitivity (i.e., testing of lower substrate concentrations), given the low solubility of oxygen in aqueous media (Garland et al., 2007). The new BD Oxygen Biosensor System (BDOBS) is a 96-well microtiter platform manufactured by BD Biosciences (Bedford, MA, USA), containing an O₂-sensitive fluorophore adsorbed onto a silicone matrix at the bottom of the wells, which offers significant potential advantages compared to Biolog for performing CLPP. As the O₂-sensitive dye–gel complex is the only component of the plates, factors such as nutrient availability, pH, or other physicochemical factors can be readily manipulated in the assay. Since the gel layer is placed on the bottom of the wells and the assay is based on fluorescence emission (no light-absorbance), it is possible to test visibly turbid (e.g., heavy soil suspensions) or opaque materials (e.g., intact soil bags) with bottom-reading plate readers. Garland et al. (2003) found that CLPP could be performed with the O₂-based system using substrate concentrations as low as 50–100 mg l⁻¹, which represent 10–100 times less than used in CLPP approaches based on either Biolog or CO₂ monitoring. The rate of fluorescent response was correlated with independent measures of *in situ* rates of substrate use, indicating that, unlike the Biolog-CLPP (Garland et al., 1997), the BDOBS-CLPP approach produced functionally relevant profiles. Specifically, rhizosphere samples from a hydroponic plant system exposed to known concentrations of either of two different types of surfactants, sodium lauryl sulfate or cocoamidopropyl betaine, showed a faster response in the BDOBS-CLPP assay to those surfactants to which the system was acclimated.

Väisänen et al. (2005) first extended the approach to soil, examining the response of different water stable aggregate size classes from a silt loam soil to a variety of substrates (including chitin, xylan, cellulose, starch, asparagine, casamino acids, mannose, sucrose, xylose, acetate, and tryptic soy broth). Detectable responses were observed for all the substrates tested, with discernable peaks in fluorescence typically occurring between 8 and 24 h of incubation. A significant positive relationship between aggregate size and activity was found for 6 of the 11 substrates

tested (cellulose, chitin, mannose, sucrose, xylan, and xylose). Cycloheximide addition significantly reduced the response to 5 of the 11 substrates (all 6 substrates above except for sucrose). Overall, these results agreed with previous studies that found greater microbial activity and fungal biomass associated with larger aggregate sizes (Guggenberger et al., 1999; Beare et al., 1997).

While these preliminary studies reinforce the potential of the BDOBS-CLPP approach, many issues remain with respect to the optimization and validation of the method for soil analysis. It is important to provide the soil microbiological research community with an understanding of the underlying capabilities/biases of the approach. One of the issues with the application of the original Biolog-CLPP approach was that it became widely applied before development of such understanding and standardization, leading to the publication of many discriminating profiles of soil communities with little knowledge of underlying causes for the differences. In other words, a lot of data was generated which provided little new knowledge. Our goal is to explore some of the methodological issues with this new form of CLPP, to provide an understanding of the approach *a priori* so that subsequent studies can provide clear, insightful results. Here we describe the first phase of this overall optimization effort, and address four major questions: 1) Can the assay be improved to detect response to lower levels of supplementation (i.e., less than the 50 mg l⁻¹ threshold observed to date) and to measure low levels of endogenous (i.e., un-amended) respiration?; 2) does the disruption of soil structure that occurs during sample processing affect the results of the assay?; 3) can the CLPP response with and without the addition of inorganic N be used as an indicator of N availability in the soil?; and 4) how does fungal activity inhibition (via cycloheximide addition) relate to independent measures of fungal to bacterial ratios?. This study emphasized the effect of various incubation conditions on the nature of the individual (i.e., univariate) fluorescent response in an effort to provide baseline information for future studies focused on multivariate profiling. A variety of soil types (organic and mineral forest soil, various agricultural soils) were tested to increase relevance to a wide range of soil microbiologists.

2. Materials and methods

2.1. Site descriptions and soil sampling procedure

Samples were collected from five soil types under different land-uses (four contrasting farming systems and a temperate forest site) (Table 1). Bulk soil samples were collected from the surface layer (0–15 cm depth) at each of three agricultural sites in Argentina, Mariana, FL and Gainesville, FL. At Eastern South Dakota Soil and Water Research Farm, soil was collected from the top 5–15 cm and about 5 kg made a composite sample. Forest soil (O-horizon material and 10 cm of mineral soil) was collected from hardwood and pine stands at the Harvard Forest Chronic Nitrogen Addition Study. Samples were collected from three replicate subplots within the control treatment plots at this site. More information about this site is provided in Frey et al. (2004). All soils were stored field moist at 4 °C no longer than 5 weeks until analysis.

2.2. Microplates and chemical supplies

The BDOBS plates (BD Biosciences, Bedford, MA, USA) were purchased from the manufacturer in the 96-well configuration. A batch of prototype 24-well microplates with a 3-ml well capacity was also supplied. The carbon sources selected for the various experiments were casein hydrolyzate from milk, L-asparagine, D-mannose, sucrose, sodium acetate and *p*-coumaric acid. Cycloheximide and the carbon compounds were analytical grade reagents from Sigma (St. Louis, MO, USA). All the stock solutions

Table 1
Physical and chemical properties of the soils studied

Location ^a	pH _w (1:2.5 w/w)	TOC (g kg ⁻¹)	Total N (g kg ⁻¹)	Extractable P (mg kg ⁻¹)	Texture ^b	USDA soil type	Predominant land use
AG	6.7	20.1	1.51	42.5 ^c	SL	Typic Argiudoll	Agriculture (cereal crops)
PF	5.5	5.2	N.D.	147 ^d	LFS	Typic Kandiudult	Pasture
DF	5	17.9	N.D.	498 ^d	Tavares sand	Typic Quartzipsamment	Uncultivated
ESD	6.9	19.3	1.77	14 ^e	Barnes SCL	Calcic Hapludoll	Agriculture (corn, soybean, wheat rotation)
HF	3.6	77.2	3.5	N.D.	Canton SL	Typic Dystrochrepts	Forest (pine and hardwood stands)

^a AG: Saldungaray, BA (Argentina); PF: Mariana, FL; DF: Dairy Farm Research Unit Gainesville, FL; ESD: Eastern South Dakota Soil and Water Research Farm, SD; HF: Chronic N Amendment Study Site Harvard Forest, MA.

^b SL: sandy loam; SCL: sandy clay loam; LFS: loamy fine sand.

^c Bray-1 P.

^d Mehlich-3 P.

^e Olsen P.

and deionized water used were filter-sterilized (<0.22 µm, Corning, Corning Inc., Germany) and stored at 4 °C before loading the plates. Solvents used in the PLFA analysis were HPLC-grade products (Merck).

2.3. Plate incubation conditions

The plates were incubated at 30 °C without shaking, and fluorescence readings were obtained from the bottom of the plate every 15 min for up to 48 h on a Dynex MFX Microplate fluorometer (Dynex Technologies, Chantilly, VA, USA) using a 485 nm wavelength excitation filter and a 604 nm wavelength emission filter. Incubation at temperature slightly higher than room temperature ensured constant temperature conditions within the heat-only microplate reader, an important consideration for temperature-dependant fluorescence.

2.4. Improvement in the sensitivity of the assay

The reversible fluorescence response reflects dissolved O₂ levels resulting from consumption via microbial activity and re-aeration via diffusion. In the attempt to increase the sensitivity of the assay by limiting O₂ diffusion into the gel layer, we tested the effect of increasing the volume of the liquid layer from 150 to 240 µl. We also examined the respiratory response in wells covered only with the regular lid vs. wells that were sealed from the atmosphere with an oxygen-impermeable film (Titer-Tops, Diversified Biotech, Boston, MA). Either 50 or 80 µl of sucrose stock solutions were added to achieve final concentrations of 0, 10, 50 and 100 mg l⁻¹. Solutions were loaded using multichannel pipette from sterile polystyrene trays into triplicate wells. The position of each C source was recorded in a template. Either 50 or 80 µl of a (NH₄)₂SO₄ stock solution was added to achieve final N concentration of 10 mg l⁻¹ in all the wells. The C and N stocks were dispensed before the soil was loaded. The respiratory response of the organic (O) and mineral (M) soil from the hardwood stand of the Harvard Forest was evaluated. Three replicates per soil layer were prepared by weighing either 10 g (M) or 5 g (O) soil in BD Falcon 50-ml conical tubes (BD Biosciences, San Jose, CA) containing 5 ml of sterile 2-mm glass beads. Twenty-five milliliters of filter-sterilized deionized water (FSDW) was added to obtain 1:2.5 and 1:5 soil:water slurries, respectively. Tubes were vigorously shaken by hand for 1 min, and the soil suspensions (either 50 or 80 µl) were immediately loaded into the plates. A single plate contained all treatment combinations and sample replicates. Organic soil sub-samples were separately treated with 5% formalin to obtain killed controls of the fluorescence response in the un-amended wells.

2.5. Comparison of endogenous activity in intact and disrupted (slurried) soil samples

In this experiment we used soil collected at the Eastern South Dakota Soil and Water Research Farm. Using BDOBS plates in a 24-well configuration, endogenous (no added substrate) activities of soil disrupted by standard sample processing were compared to those of intact soil samples that remained confined in small mesh bags during incubation. Soil microbags (ca. 1 cm³) were constructed of two layers of nylon spawn sac netting (Atlas-Mike's Bait Co, Ft. Atkinson, WI) that was filled with 2.0 ± 0.1 g of field moist, sieved (<4.75 mm), homogenized soil and sealed with elastic poly thread (Atlas-Mike's Bait Co.). The soil microbags were buried (8 June 2007) in a 10-cm deep trench in the same fallow field where the soil was originally collected. After 160 days (15 November 2007), the microbags were excavated and returned to the lab for analysis. Ten intact soil microbags were placed directly in the wells of the plate with 2.4 ml of FSDW to fill the well. Another 10 microbags were disrupted by emptying the contents of each bag into a sterile test tube with 2 ml of FSDW, adding the empty bag, and shaking (30 s) vigorously with three, 6-mm ceramic beads. The resulting slurry with empty bag was poured into a microplate well and topped with 0.4 ml FSDW. The plate was sealed with sterile, poly adhesive tape and fluorescence readings collected. Three positive controls (100 mM NaS) and one negative control (sterile water) were run per plate. Additional sets of 10 intact and 10 disrupted bags were analyzed after 1, 11, and 33 days storage (4 °C) to determine if storage interacted with sample disruption.

2.6. Response to different amendments

2.6.1. Nitrogen effects on substrate response

A microcosm fertilization experiment was designed in order to study how temporal changes in N availability affect the microbial respiratory response in the plates. Soil used in this experiment was collected from the Dairy Farm Unit in Gainesville, FL and taken to the lab where it was split in two and either treated with inorganic fertilizer or left untreated. Three kilograms of soil were thoroughly mixed with 200 ml of a salt solution containing 535.2 mg NH₄NO₃, 378.8 mg KNO₃ and 102 mg KH₂PO₄. Untreated soil received the same volume of deionized water. Twelve 20 g-soil samples were taken from each of these treatments, placed in 50 ml-screw-capped plastic tubes, and incubated in the dark in a growth chamber set at 28 °C. Triplicate soil microcosms were destructively sampled 1, 5, 8 and 19 days after treatment. Soil suspensions were prepared with 10 g soil sub-samples as described in Section 2.4 and 160 µl of a 1:2.5 soil:water slurry was loaded per well. Plates were pre-loaded

with 40 μl of 300 mg l^{-1} stock solutions of C sources (mannose and asparagine), a concentration designed to deliver 50 mg l^{-1} in the wells with 240 μl final volume. Similarly, stock solution of $(\text{NH}_4)_2\text{SO}_4$ was designed to dispense 10 mg l^{-1} (N10) when adding 40 μl in the wells. Assuming that a completely homogeneous soil slurry is inoculated into each well, the above-mentioned concentration on diammonium sulfate provided $\approx 8 \mu\text{g N g}^{-1}$ soil. Control, un-amended wells were loaded with equivalent volumes of FSDW to measure the response to background C with or without N addition in the plate (N0).

2.6.2. Cycloheximide effects on substrate response

The aim of this experiment was to evaluate if the BDOBS can be used with selective inhibitors of eukaryotic activity to assess the relative contribution of bacteria and fungi to CLPP in a given soil sample. To address this question, we correlated the respiratory response with measurements of fungal:bacterial ratios. We examined the effects of cycloheximide on the fluorescence response in the un-amended control and following the addition of coumaric acid, casein, asparagine, sodium acetate, and mannose. We tested the pasture, agricultural and forest soils in this experiment. For the latter, only the mineral soil from the pine stand was tested. Triplicate soil samples (10 g) from each site were weighed and suspended in FSDW to obtain 1:2.5 soil:water slurries as described above (Section 2.4), and 80 μl of soil suspension was loaded per well. Plates were pre-loaded with 80 μl of substrate stock solutions (150 mg l^{-1}) prepared to deliver 50 mg l^{-1} in wells with 240 μl as the final volume. Cycloheximide was added to the wells by loading 80 μl of the following filter-sterilized stock solutions: 0, 8 (low) and 32 (high) mg ml^{-1} , designed to get a final concentration of 0, 2.7 and 10.7 mg ml^{-1} in the wells, respectively. Assuming that a completely homogeneous soil slurry is inoculated into each well, the above-mentioned stock solutions provided 0, 20 and 80 $\text{mg cycloheximide g}^{-1}$ soil, respectively.

2.7. PLFA analysis

We determined the fungal:bacterial ratio in soil samples by phospholipid fatty acid analysis. Phospholipids in freeze-dried soil were extracted and quantified according to the procedure of Bossio and Scow (1998). Triplicate samples (5 g for agricultural soil and 1 g for forest soil) were extracted for 3 h with a one-phase extraction mixture containing chloroform:methanol:phosphate buffer (1:2:0.8 v/v/v). Phospholipids were separated from neutral lipids and glycolipids using silica gel column chromatography. The polar lipid fraction was derivatized into fatty acid methyl esters (FAMES) using mild-alkaline methanolysis. Samples were analyzed by capillary gas chromatography on a Varian CP-3800 Gas Chromatograph (Varian, Inc.), equipped with a flame ionization detector (FID), column Agilent HP-5ms (Agilent Technologies, Palo Alto, CA), and autosampler CP-8410. A 5 μl injection was analyzed at an initial temperature of 150 $^{\circ}\text{C}$, ramped to 312 $^{\circ}\text{C}$ at 3 $^{\circ}\text{C min}^{-1}$. Peaks were identified using bacterial fatty acid standards (Matreya LLC, Pleasant Gap, PA, USA). The fatty acids i15:0, a15:0, i16:0, 16:1 ω 7c, 16:1 ω 7t, i17:0, cy17:0, 18:1 ω 7c, cy19:0 were used to represent bacterial PLFAs (Leckie et al., 2004), and 18:2 ω 6c, 18:1 ω 9c were used as markers of fungal biomass (Waldrop and Firestone, 2004). The ratio of fungal PLFAs to the sum of bacterial PLFAs in soil was calculated as an index of the relative proportion of fungi and bacteria (F:B).

2.8. Fluorescence data analysis

Fluorescence response was converted to normalized relative fluorescence units (NRFU) by dividing the readings at each time point by the response at 1 h. The time to minimum threshold

response (i.e., time for NRFU to increase by 10%) was also calculated (Garland et al., 2003). The elapsed time to a minimum NRFU of 1.5 and the integrated area of NRFU vs. time at 30 h were calculated for data from the 24-well BDOBS plate format. Fluorescence response vs. incubation time was plotted, and integrated area calculated using Sigmaplot v. 6 (Systat Software, Inc., San Jose, CA, USA). One-Way ANOVA was performed to test the significance of N and cycloheximide effects on different parameters of fluorescence response curves. Whenever the assumption of normality was not met, Kruskal–Wallis One-Way ANOVA on Ranks was used. A two-way ANOVA was performed to test for main effects of sample disruption and storage time (see Section 2.4). Pairwise comparisons were tested for significance at $P=0.05$ using Tukey–Kramer test. Correlation analysis was performed to explore the relationship between the degree of inhibition in BDOBS responses with cycloheximide treatment, and independent measures of F:B ratios in the samples.

3. Results

3.1. Improvement in sensitivity of the plate assay

Wells filled with 240 vs. 150 μl showed a greater response (i.e., higher maximum and integrated fluorescence), reflecting the larger amount of O_2 consumption resulting from the higher total amount of carbon added (Fig. 1). However, when equal amounts of substrates were added in different volumes in a separate test, the 240 μl wells still exhibited a faster response, indicating that O_2 diffusion to the gel layer was reduced due to changes in water depth or a deeper layer of settled soil particles on the gel layer (results not shown). Covering the wells had no effect on the initial rate, but did increase the maximum response and extended the fluorescence. The response to the highest sucrose concentration at least doubled that observed for the 50 mg l^{-1} . The peak decreased with decreasing sucrose concentration, although even at the lowest dose (10 mg l^{-1}) the amended response was discernable from endogenous (un-amended) respiration. Fluorescence caused by endogenous respiration in the un-amended control ranged from just above 1.05 to 1.15 in the 240 μl wells, with higher values in the C-rich O-horizon. A slight variation in signal present in the killed controls was most likely due to a lag in the heating of the wells to 30 $^{\circ}\text{C}$ given temperature sensitivity of the fluorescence (Fig. 1).

3.2. Comparison of endogenous activity in intact and disrupted (slurried) soil samples

There were significant main effects for sample disruption ($P<0.001$), storage time ($P=0.003$) and the interaction term ($P=0.017$) on elapsed time to an NRFU of 1.5. The pairwise comparisons showed that intact samples took longer to reach NRFU 1.5 than disrupted samples under all storage conditions ($P<0.001$, 16 pairwise comparisons). However, the significant main effects of storage time and on the interaction term were due to just two of the remaining 12 pairwise comparisons, which showed that the intact bags after 11 days of storage had a significantly delayed response compared to intact bags at 0 day ($P=0.001$) and 1 day ($P=0.014$) of storage. There were also significant main effects for sample disruption ($P=0.016$), storage time ($P=0.016$), and the interaction term ($P=0.006$) for integrated area of NRFU vs. time at 30 h. However, these main effects were due to only 5 of the 28 pairwise comparisons and did not show consistent differences associated with sample disruption or storage time. Four of the significant comparisons involved intact bags after 11 days storage which had a significantly smaller area than disrupted bags after 1, 11, and 33 days storage and intact bags after 1 day storage. Intact bags at 33 days storage also had a significantly smaller area than intact bags

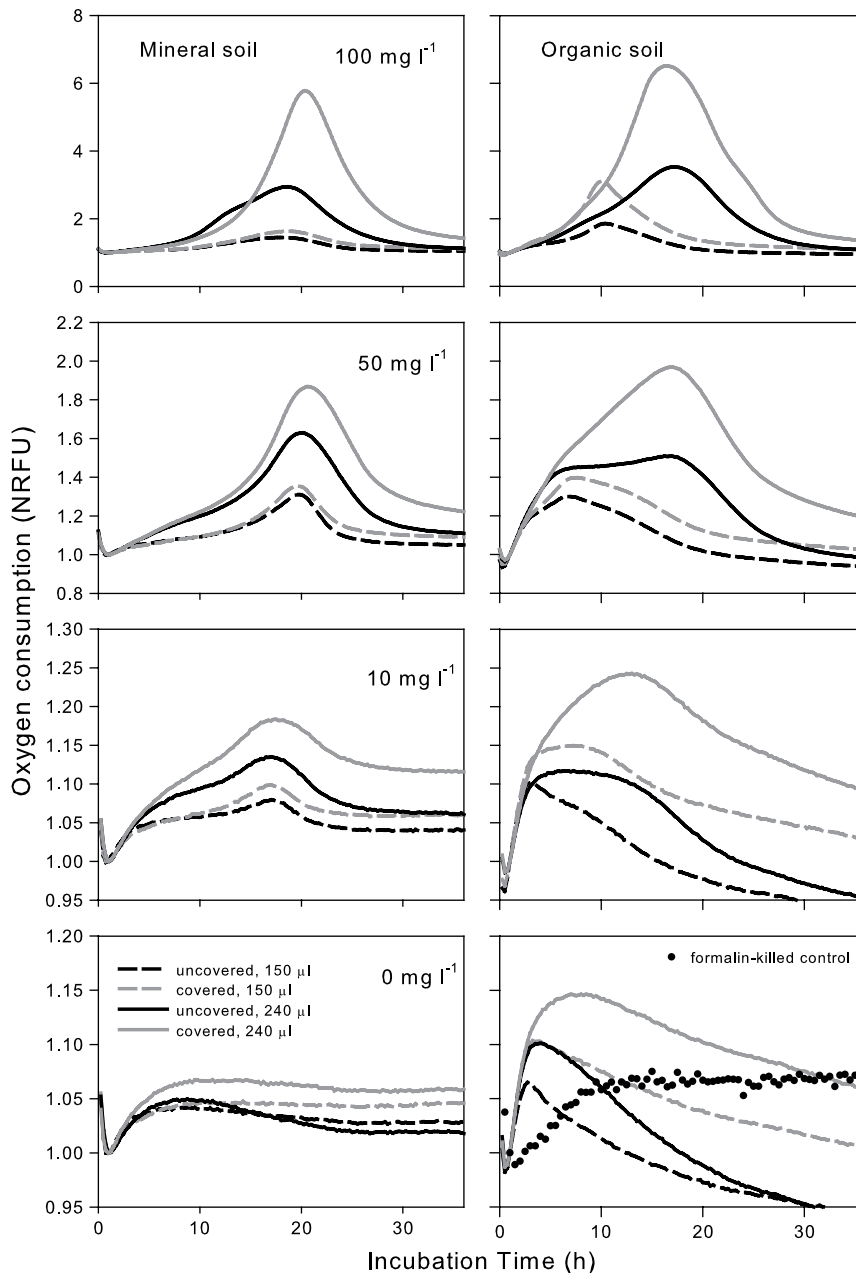


Fig. 1. Oxygen consumption response curve of mineral and organic soil horizons from Harvard Forest, amended with different doses of sucrose (10, 50, 100 mg l⁻¹) or un-amended. Dots show the temperature-dependant fluorescence response of the formalin-killed control in the O-horizon. Error bars are not shown for clarity of presentation.

after 1 day storage. While intact bags had a longer lag than disrupted bags, they exhibited a sharper rise in activity and a comparable peak of activity that resulted in equitable total activities over a 30 h time frame with the disrupted bags. Intact bag replicates were more variable than disrupted bag replicates. Storage did not affect the activities of disrupted bags, but intact bags showed a gradual decrease in activity with storage time.

3.3. Response to amendments

3.3.1. Nitrogen effects on fluorescence response

The use of amended mannose increased in N10 vs. N0 treated wells at all sampling days (Fig. 2). In contrast, no effect of nitrogen amendment on mannose response was observed at Day 1 in the fertilized microcosms. On following sampling days, N10 addition increased the peak in fluorescence in comparison to the N0 wells. While the fluorescence response to asparagine showed no differences

between N0 and N10-amended wells in the control microcosms during the entire assay, fertilized microcosms exhibited a slight increase in the fluorescence response to asparagine when amended with N10 in the plate, up to Day 8 (data not shown). The fluorescence response in the un-amended wells did not differ between N0 and N10 amendments neither in the fertilized nor in the control microcosms at Day 1 (Fig. 3). On Day 5, the N10 amendment induced a higher response in un-amended wells both in the control and fertilized microcosms. Similar fluorescence responses in the un-amended wells were observed on following sampling days for both the control and fertilized microcosms, except for the slight decrease in the extent of the response in the fertilized soil with increasing incubation time.

3.3.2. Cycloheximide effects on fluorescence response

The treatment with cycloheximide consistently retarded the rate of fluorescence response of several substrates. When looking at time to minimum (Fig. 4), a high dose of cycloheximide

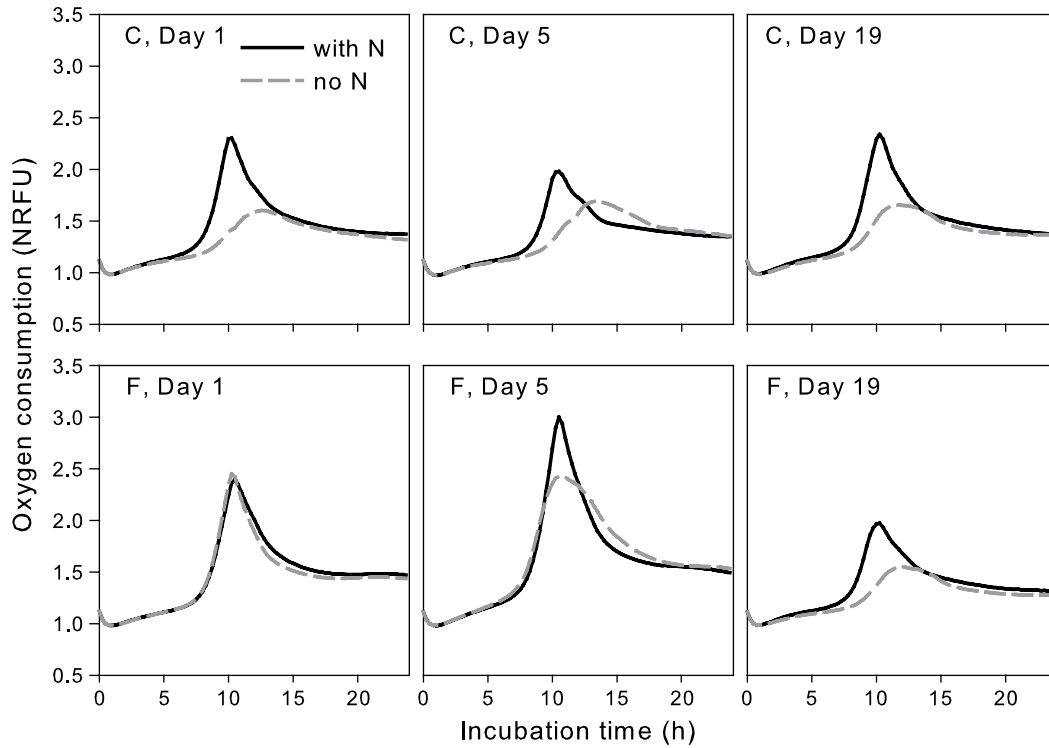


Fig. 2. Oxygen consumption curves of the uncultivated soil from Dairy Farm, fertilized (F) or control (C) soil microcosms amended with mannose (50 mg l^{-1}) and with 10 mg l^{-1} diammonium sulfate ($\approx 8 \mu\text{g N g}^{-1}$ soil) or without N. Error bars are not shown for clarity of presentation.

(10.7 mg ml^{-1}) delayed the initial increase in response of casein, acetate and asparagine in two soils (pasture and agricultural land-uses), while it had no significant effect on coumaric acid. The time to minimum of the un-amended control was stimulated by the

addition of low and high rates of fungicide in these soils. In the forest soil, the high rate of cycloheximide increased time to minimum to coumaric acid, casein, and acetate (the latter was not statistically significant).

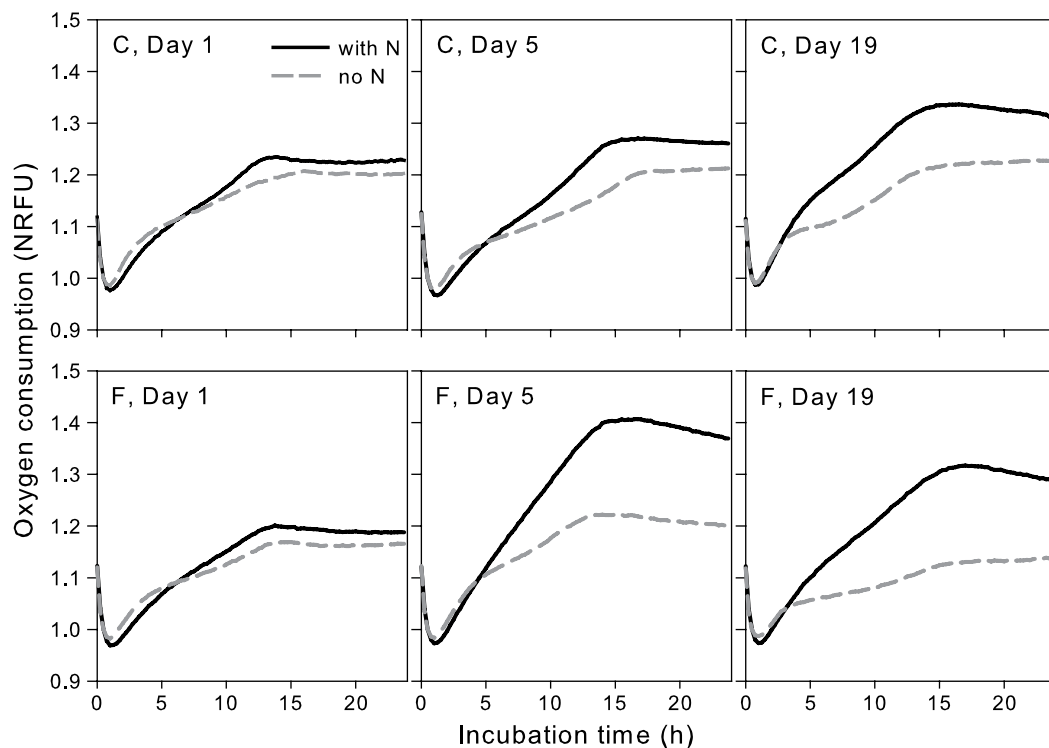


Fig. 3. Oxygen consumption curves of the uncultivated soil from Dairy Farm, fertilized (F) or control (C) soil microcosms un-amended (i.e., no C added) and with 10 mg l^{-1} diammonium sulfate ($\approx 8 \mu\text{g N g}^{-1}$ soil) or without N. Error bars are not shown for clarity of presentation.

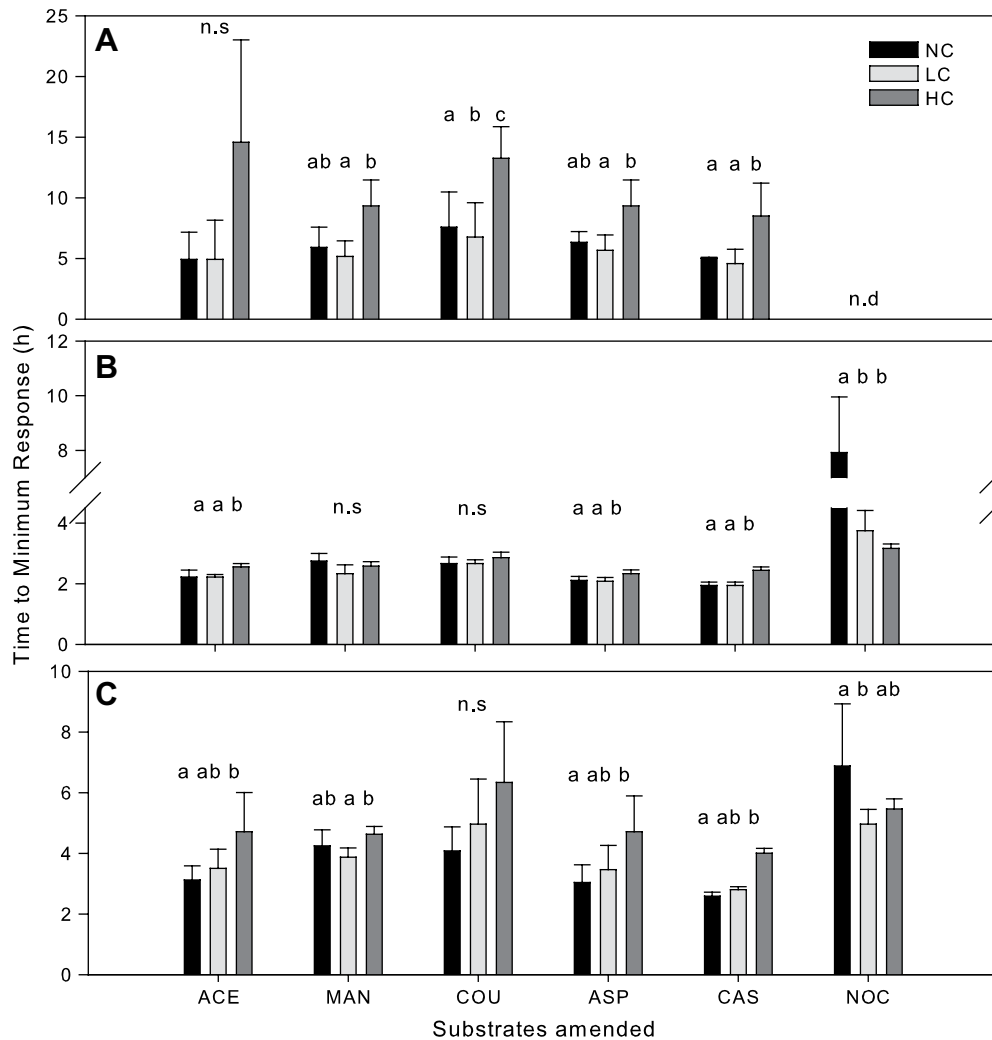


Fig. 4. Mean time to minimum response for added C-substrates with different levels of cycloheximide. NC = 0 mg g⁻¹ soil, LC = 20 mg g⁻¹ soil, HC = 80 mg g⁻¹ soil. A: forest soil (HF); B: agricultural soil (AG); C: pasture soil (PF). Bars represent mean \pm S.E. ($n = 3$). Different letters show significant differences among cycloheximide levels within substrates (Tukey–Kramer Test, $P < 0.05$).

Assuming that time to minimum without cycloheximide (TMR) addition represents the delay in initial response of the overall microbial community (mainly fungi and bacteria) and time to minimum with cycloheximide (TMR_{cyclo}) represents the delay of the surviving fraction (mainly bacteria) in utilization of the amended substrate, we calculated the following activity ratio (AR) for each substrate: $(TMR - TMR_{cyclo})/TMR_{cyclo}$, which should reflect the F:B respiratory responses in the BDOBS plates. An AR value equal to zero should be interpreted as no delay in the initial response with cycloheximide, thus the amended substrate is not being used by fungi in the plate. Negative AR values would mean that cycloheximide delayed the initial response to a given substrate to some extent. Overall AR values varied between 0 and -0.667 in soils with F:B PLFA ratios at a range of 0.12–0.23 (data not shown). No correlations were found between AR and F:B PLFA ratio for any of the substrates tested (Table 2).

4. Discussion

While previous work has demonstrated that the BDOBS system allows for testing substrate concentrations 10–100-fold lower than other substrate-induced respiration approaches (Väisänen et al., 2005), we were able to further improve its sensitivity. Garland et al. (2003) working with hydroponically grown wheat rhizosphere

samples reported that levels of substrates as low as 50 mg l⁻¹ yielded clear, well defined and easy to interpret response curves, but they were unable to detect a response with lower concentrations (i.e., 5 and 10 mg l⁻¹). Our results showed that substrate concentrations as low as 10 mg l⁻¹ can be readily detected as long as larger liquid volumes (240 μ l well⁻¹) are loaded; the effects of plate sealing were minimal and mainly were associated with delayed re-aeration of the plates. The increased sensitivity resulting from larger liquid volumes is probably caused by a combination of the thicker layer of settled particles, and a greater total amount of carbon available to the settled layer.

The amendment with lower levels of substrates may lessen the selective enrichment of copiotrophic microorganisms, minimize

Table 2
Correlation between activity ratio $[(TMR - TMR_{cyclo})/TMR_{cyclo}]$ for different substrates and F:B PLFA ratio

	Acetate	Coumaric acid	Mannose	Asparagine
r^a	0.47	0.67	0.22	0.08
R^2	0.22	0.43	0.06	0.01
F-value	1.74	4.60	0.39	0.04
P-value	0.24	0.08	0.55	0.84

^a Correlations were calculated for eight ($N = 8$) soils. Each TMR value is the average of $n = 3$ replicates per substrate; the TMR with cycloheximide refers to high cycloheximide level.

physiological substrate shock and changes in controlling parameters (pH, osmotic pressure, etc.), while still providing a measurable response of microbial activity. Brant et al. (2006) reported that low levels of ^{13}C -labeled substrate additions ($50\ \mu\text{g C g}^{-1}$ soil) did not enrich for any particular component of the soil microbial community, as evidenced by ^{13}C -PLFA profiles. Assuming that the most concentrated soil slurry used was evenly mixed when loaded into the wells, we estimated that $\geq 75\ \mu\text{g}$ of substrate was added per gram soil with the lowest concentration of C source and highest liquid volume. Although the limited incubation time (6 h) of the SIR approach ensures measurement of activity of microbes already present in soil rather than growth, high substrate concentration (10–100 mM) (Degens and Harris, 1997; Campbell et al., 2003) may cause acute toxicity or inhibition of oligotrophic bacteria and may not reflect *in situ* C availability (Hu et al., 1999).

The BDOBS was also sensitive to detect background levels of respiration, especially in organic vs. mineral soil horizons, which may mainly reflect use of indigenous soil organic carbon (SOC). Basal respiration is commonly measured as the release of CO_2 or the uptake of O_2 , by several techniques such as gas chromatography, electrodes, manometers, respirometers or titration (Pell et al., 2006). The MicroResp system acts essentially as a micro-respirometer and may also be used to measure basal respiration (Chapman et al., 2007). Further studies are needed to correlate the BDOBS measurement of basal respiration to other measures of either microbial respiration or labile SOC.

The use of greater soil amounts in the 24-well plates may be an advantage for study of endogenous respiration, low substrate concentrations, and use of soil microbags, but differently shaped response curves may be produced compared to 96-well plates. Well geometry, soil mass, ruthenium dye mass, and other additive masses will affect the kinetics of oxygen consumption and production and consequently the shape of the response curve and the selection of the appropriate analysis parameters. Resulting curves for either plate format will generally have three characteristics for consideration: lag, slope, and maximum. Time to minimum parameter accounts for the lag phase while integrated area (at a point nearing or at maximal response) captures all three characteristics, although trade-offs may occur and so it may be useful to separately analyze maximal and calculated or modeled slope values. The analytical approaches used on response variables for other CLPP approaches should all be applicable, each with their distinctive set of advantages and limitations depending on the situation (Preston-Mafham et al., 2002; San Miguel et al., 2007).

Laboratory activity measurements frequently necessitate mixing of a substrate with the sample and use detection methods that require distribution and/or diffusion of the product. Therefore, many standard activity protocols require mixing soil samples with water or buffer and using the resulting slurry to inoculate a microplate or microcosm. The loss of soil structure and redistribution of nutrients caused by making a soil slurry are fundamental limitations that may produce artifactual results. The response curves for intact bags were generally sigmoidal while the disrupted bags were more linear and less variable within and between sample sets. We found significant differences in the lag phase of activities between intact and disrupted soils, but similar levels of integrated or maximal activities. The limited effects of sample disruption probably resulted from the short incubation period and low selective pressure associated with not adding any nutrients. The duration of sample storage had no significant effect on the activity of disrupted samples in the BDOBS plates.

One of the important attributes of the BDOBS approach is the ability to readily manipulate assay conditions such as inorganic nutrient levels, therefore aiding in the understanding of factors limiting activity in the soil. Brown et al. used the BDOBS approach to examine nutrient limitations and patterns of use of soluble

carbon sources of microbial communities in a low pH, sandy soil in a Florida scrub oak ecosystem. These authors reported that N and P limitations were preventing the microbial use of available C in the system. However, this study was done in an extremely nutrient-limited scrub oak soil in Florida, and thus may have little relevance for agricultural systems. We further examined the fluorescence response to N amendment in BDOBS plates and related the response to *in situ* N content of soil by manipulating this nutrient in soil lab incubations. The microcosm experiment showed that this approach was sensitive to detect temporal changes in N availability in soil. While inorganic fertilizer provided N to sustain both native C and amended-substrates use shortly after the treatment, the effect on mannose started diminishing on Day 5 after addition. The fertilized microcosms also showed a higher response in the un-amended control with N added in the plate. This has two plausible explanations: 1) enrichment of nitrifying bacteria has occurred in these microcosms, therefore increasing O_2 uptake for this aerobic reaction when N was amended (Shi and Norton, 2000); 2) increased degradation of SOC (priming) caused by the addition of mineral fertilizer (Kuzuyakov et al., 2000). The lack of response to asparagine of untreated soil microcosms is consistent with the idea that the use of this amino acid by microbes should not show N-dependent response. We attempted to couple the use of the eukaryotic protein synthesis inhibitor cycloheximide with the BDOBS assay to study the soil community structure by assessing relative use of substrates by bacteria and fungi. Väisänen et al. (2005) provided preliminary evidence of this approach, but no independent measures of fungal and bacterial biomass/activity were collected. We conducted a more thorough evaluation of this topic by evaluating BDOBS responses with two cycloheximide concentrations in three different soil types. Cycloheximide consistently retarded the initial response of several substrates (casein, asparagine, acetate, coumaric acid) in most of the soils tested. In contrast, Väisänen et al. (2005) reported that these compounds were preferentially used by bacteria, but they added 10 times less cycloheximide and phosphate buffered mineral salts solution (PBMS) to soil slurries in the plates. It has been reported that fungi may regulate amino acid uptake by transporters that can be repressed by presence of ammonia in the culture media (Bapat et al., 2006). Thus, it is possible that the N limitation imposed in our study aided in the discrimination of C sources that could be preferred by fungi in soil under natural conditions. It has been reported that respiration measurements should be restricted to the first 8–10 h of soil incubation to avoid confounding changes in microbial biomass (i.e., newly formed biomass contributing to respiration, non-inhibited part of biomass taking over the respiration of the inhibited part, and/or mineralization of cycloheximide by surviving biomass) (Velvis, 1997). The rapid estimate of lag used in this study (i.e., time to minimum) seems to be a suitable parameter given its typical value of 3–8 h. Väisänen et al. (2005) used time-to-peak to evaluate the effect of cycloheximide on the respiratory response to eleven C sources using the BDOBS. However, this parameter can be confounded since it takes a longer time to reach a higher peak, and it is difficult to calculate when sharp peaks do not appear.

The correlation analysis found no agreement between the extent of the delay in response and estimates of fungal biomass based on PLFA analysis. Similarly, Raubuch et al. (2006) found no correlation between the relative decrease in ATP content after cycloheximide addition and the ergosterol-to-microbial biomass C ratio, which is an indicator for the fungal proportion of the total microbial biomass. Bååth and Anderson (2003) also reported a lack of congruence between the fungal/bacterial ratios estimated with the PLFA method and the selective respiratory inhibition technique. In addition, we observed that high cycloheximide addition caused an earlier fluorescence response in the un-amended wells in two of the three soils, suggesting that rapid turnover of killed fungal

biomass is another potential confounding factor. These and other limitations of the selective respiratory inhibition technique have been reported before (Imberger and Chiu, 2001; Nakamoto and Wakahara, 2004; Susyan et al., 2005). Overall, we conclude that the delay in time to minimum resulting from cycloheximide addition may be useful for assessing the role of fungi in the use of different substrates, but should not be used to assess overall fungal activity in the sample.

5. Recommendations and conclusions

We were able to further increase the sensitivity of the BDOBS to detect low levels of substrate amendment and even un-amended (basal) respiration in soil slurries by simply increasing the final volume from 150 to 240 $\mu\text{l well}^{-1}$. Even though covering the plates caused a relatively higher peak and larger response by retarding the re-aeration of the plate, we suggest that it is not necessary to seal the plate to improve the detection of respiration if a large volume of liquid is used. As low as 10 mg l^{-1} of substrate produced a clear fluorescence response. However, when the objectives of the experiments were to assess the effects of N or cycloheximide amendments on the fluorescence response, we selected 50 mg l^{-1} of substrate as to more clearly discern shifts in response. The disruption of soil structure necessary to prepare a soil slurry had no effect on overall fluorescence response as compared to the response of intact soil samples enclosed in microbags. The use of BDOBS was successfully used to detect N limitations. Finally, the coupling of the BDOBS with the use of the fungal inhibitor cycloheximide allowed for the discrimination of the C sources that would be preferentially used by fungi in natural environmental conditions, but the degree of reduction in respiration due to cycloheximide addition could not be used to directly estimate fungal to bacterial ratios. Based on these results, we provide an outline of our recommended protocol (published as [Supplementary material](#) on the online version of this article).

The ability to continuously monitor respiratory response to very low substrate levels is a clear advantage of this approach relative to other CLPP approaches. Comparative studies of the newer CLPP approaches (i.e., BDOBS, MicroResp, Multiple SIR) would be useful, particularly those involving experimental manipulation of soil functional activities. The BDOBS system could be used in several applications other than CLPP, including basal respiration measurements, ecotoxicological studies (e.g., pollution-induced community tolerance to toxicants), and the measurement of other oxygen-consuming reactions (e.g., O_2 consumption by nitrifying bacteria). Finally, molecular profiling of the active microorganisms within the wells may prove to be a useful tool for screening the factors which activate different fractions of the soil microbial community, thereby leading to better understanding of the linkages between community structure and function in soil communities.

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Appendix Supplementary data

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.soilbio.2008.08.015](https://doi.org/10.1016/j.soilbio.2008.08.015).

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