Soil Biology & Biochemistry 47 (2012) 46–52

Contents lists available at SciVerse ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Examining N-limited soil microbial activity using community-level physiological profiling based on O_2 consumption

J.L. Garland [a,1], M.C. Zabaloy [a,*], M. Birmele [a], C.L. Mackowiak [b], R.M. Lehman [c], S.D. Frey [d]

[a] Dynamac Corporation, Kennedy Space Center, FL 32899, USA
[b] University of Florida, North Florida Research and Education Center Quincy, FL 32351-5677, USA
[c] USDA-ARS-North Central Agricultural Research Lab, Brookings, SD 57006, USA
[d] Department of Natural Resources and the Environment, University of New Hampshire, Durham, NH 03824, USA

ARTICLE INFO

Article history:
Received 12 July 2011
Received in revised form
13 December 2011
Accepted 16 December 2011
Available online 29 December 2011

Keywords:
Community level physiological profiling
Bioavailable N
N limitation
Enrichment

ABSTRACT

Nitrogen-limited soil microbial activity has important implications for soil carbon storage and nutrient availability, but previous methods for assessing resource limitation have been restricted, due to enrichment criteria (i.e., long incubation periods, high substrate amendments) and/or logistical constraints (e.g. use of radioisotopes). A microtiter-based assay of basal and substrate induced soil respiration based on O_2 consumption may be a rapid, ecologically relevant means of assessing N limitation. The present study evaluated this approach by examining 1) the extent and duration of N limitation on soil respiratory activity following different levels of N fertilization in the field, and 2) the relationship between N-limited activities and growth under the assay conditions. Fertilization rate and the time since fertilization had significant impacts on the degree of N limitation of soil microbial activity. The highest fertilization rate showed the earliest and most persistent reduction in N limitation, as would be predicted from the higher concentration of extractable inorganic soil N observed with this treatment. Bacterial growth under the assay conditions, as estimated by quantitative-PCR of 16S rRNA genes, was less than twofold in soils demonstrating a rapid respiratory response (i.e. peak within 6–8 h of initiating incubation) to up to fourfold in soils demonstrating a slower respiratory response (i.e., peak response after ~14 h of incubation). Increased respiratory response with N amendment was usually associated with increased cell growth, although for rapidly responding soils some C sources showed N-limited use without growth. This was likely due to exhaustion of the relatively low levels of available C amendment before growth was detected. The method appears useful for assessing N-limited microbial growth, and it may be effective as a rapid indicator of bioavailable soil N. It may also be a tool to evaluate the complexity of N limitation among various metabolic pathways found in soil microbial communities, particularly if linked to dynamics in community structure and gene activation.

1. Introduction

The relative importance of carbon (C) and nitrogen (N) limitation on microbial activity in soils has significant consequences for effective agricultural nutrient management and development of potential strategies to enhance soil C sequestration. While C is often considered the primary factor limiting soil microbial activity (Nordgren, 1992; Demoling et al., 2007), N availability has an important influence on the fate of processed C. The traditional view emphasized the linkage between respiration and growth, leading to definition of C/N thresholds which control the relative amounts of microbial N immobilized or mineralized. Recent work has examined the effects of the uncoupling of respiration from growth on C and N processing in soil (Schimel and Weintraub, 2003). Studies in chemostat culture (Tempest, 1978) and in the field (Manzoni et al., 2008) have demonstrated that the efficiency of microbial utilization of C decreases under nutrient-limited conditions. Decreased efficiency can involve several mechanisms, including incomplete oxidation of substrates (i.e., overflow metabolism), uncoupled oxidation of NADH from ATP production (e.g., branches in the electron transport chain), and decreased membrane resistance

* Corresponding author. Present address: Center of Renewable Resources of Semiarid Region (CERZOS), Department of Agronomy, South National University (UNS), San Andres 800, 8000 Bahía Blanca, Argentina. Tel.: +54 291 4595126; fax: +54 291 4595127.
E-mail address: mzabaloy@uns.edu.ar (M.C. Zabaloy).
[1] Present address: National Exposure Research Laboratory, Office of Research and Development, United States Environmental Protection Agency, 26 W. Martin Luther King Jr. Drive, Cincinnati, OH 45268, USA.

0038-0717/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2011.12.016

resulting in reduced efficiency of ATP generation via chemi-osmosis (i.e. energy spilling) (Tempest, 1978; Russell and Cook, 1995; Teixeira de Mattos and Neijessel, 1997; Liu, 1998). The potential selective advantage of "wasting" energy is the maintenance of ATP levels and other precursor molecules to enable rapid initiation of growth once N (or other nutrients) becomes available (Russell and Cook, 1995). A similar rationale of "metabolic alertness" has been invoked to explain the rapid increase in respiration by soil microorganisms in response to low levels of C substrates (De Nobili et al., 2001).

The most commonly employed approach to assess N limitation involves measuring the difference in substrate induced respiration (SIR) of soil samples with and without N supplementation (Nordgren, 1992; Dilly, 1999; Gnankambary et al., 2008). Carbon dioxide is monitored during 5–10 days of incubation following addition of approximately 30–400 mg C g^{-1} soil and small amounts of N, typically 0.3 mg g^{-1} soil (Gnankambary et al., 2008) to 2.5 mg g^{-1} soil (Nordgren, 1992) to ensure that growth occurs. This approach is limited because it indirectly assesses growth via CO_2. It is also limited in that it uses selective enrichment and induction of N limitation resulting from high amounts of C amendment. Direct measurement of growth via leucine or thymidine incorporation using much lower levels of C amendment (<3 mg C g^{-1} soil) provides a more effective means of assessing C and nutrient limitation on growth (Demoling et al., 2007). Cell production and/or respiration assays based on radiolabeled substrates are effective tools to evaluate the effects of C and N on microbial activity (Thiet et al., 2006; Demoling et al., 2007; Rinnan and Baath, 2009), largely because they allow for physiological assessment of minimally enriched communities using ecologically relevant substrate levels. Garland et al. (2003) introduced a simple yet versatile platform for assessing microbial respiration which shares some of the aforementioned strengths. This technique builds on the community-level physiological profiling (CLPP) approach introduced by Garland and Mills (1991) by replacing the Biolog redox dye chemistry with an oxygen-sensitive fluorophore. As with Biolog and many other laboratory activity measurements, this method still requires mixing soil samples with water and using the resulting slurry to inoculate the microplate. However, more dense slurries can be pipetted in the wells since turbidity does not interfere with the bottom reading of fluorescence. Previous experiments have shown that disruption of soil structure had no effect on overall fluorescence response as compared to the response of intact soil samples enclosed in microbags (Zabaloy et al., 2008). The new method reduces selective enrichment bias by detecting substrate-induced respiration in soils at amendment levels two to three orders of magnitude lower than those required in Biolog plates ($\sim$100 μg C g^{-1} soil), while also allowing for rapid assessment of basal soil respiration (Zabaloy et al., 2008). Since the new assay does not employ any proprietary nutrients, incubation conditions (e.g., available N) can be readily manipulated. Therefore, the assay allows for rapid assessment of C (in a multitude of substrates) and N amendments on microbial respiration. Preliminary studies have generally reported stimulatory effects of N supplementation on soil respiration using the assay (Väisänen et al., 2005; Brown et al., 2009). However, recent work found that the difference in oxygen consumption in soils with and without N supplementation (N_{diff}) during short-term ($\leq$8 h) incubations transiently decreased following fertilization of laboratory microcosms (Zabaloy et al., 2008) and agricultural fields (Garland et al., 2010).

In this work we demonstrate the link between soil respiratory response and N by measuring basal and substrate-induced respiration with and without added N in agricultural soils collected over the growing season from replicated plots receiving different N application treatments. Using three different soils, we further investigate this N-dependent respiratory response and its relationship to cell growth in the assay by measuring growth with quantitative PCR (rRNA genes) during the activity-based assay.

2. Materials and methods

2.1. Field study: site description, soil sampling and analysis

Experimental field plots were located on the Eastern South Dakota Soil and Water Research Farm, Brookings, SD (44.32° N; 96.77° W). The 65-ha farm is operated by the North Central Agricultural Research Laboratory (USDA-ARS-Northern Plains Area). The research farm is located in the upper Midwest corn-belt in the transition zone (mixed grass prairie) between the cool (frigid temperature regime) and warm (mesic temperature regime) prairie of the northern Great Plains (Pikul et al., 2001). The site is located at 500 m elevation in a sub-humid climate (58 cm average annual precipitation) with an average annual temperature of 8 °C. Site topography is nearly level. Soil characteristics are summarized in Table 1. Triplicate plots of continuous dry land corn (*Zea mays* L.) were planted and managed under conventional tillage (fall chisel plow, spring disking) with a uniform annual regime of chemical weed control. The plots were established in 1990 within a randomized block design containing other rotational treatments (Pikul et al., 2001). Each of the replicate corn plots were split into three subplots (30 m × 30 m) that received: 1) high N (HN, fertilized for a grain yield goal of 8.5 Mg ha^{-1}); 2) medium N (MN, fertilized for a grain yield goal of 5.3 Mg ha^{-1}); and 3) low N (LN, no fertilizer). Wheel traffic was controlled on all plots. A starter fertilizer (N, P, K) was applied at the time of seeding (May 8, 2008) for all plots with urea (15.7 kg N ha^{-1}) in dry form placed 5 cm below the soil surface and 5 cm to one side of the seeds. The bulk of the N fertilizer was applied to the medium (65 kg N ha^{-1}) and high (125 kg N ha^{-1}) N plots at mid-season (June 20, 2008) by broadcasting dry urea and incorporating it into the soil (10 cm).

Soil samples (3.2-cm diameter probes, 0–15 cm depth) were collected from eight locations arranged in an "X" pattern within each subplot and bulked. Soil samples were collected on 05 May, 2008 (preplant, −45 days from fertilization [DFF]), 30 June (10 DFF), 14 July (24 DFF), 11 August (53 DFF), and 20 October (123 DFF). Soil samples were briefly homogenized and split in two, and subsamples were sent by overnight courier on ice to Kennedy Space

Table 1
Soil properties of the study sites.

Site	pH_w	OM[a] (%)	TKN[b] (mg kg^{-1})	Texture	USDA class	Land use
Brookings (SD)	6.9[c]	4	1770	Barnes sandy clay loam	Calcic Hapludoll	Agricultural (corn)
Hague (FL)	4.8[c]	3.0	1400	Tavares sand	Typic Quartzipsamment	Agricultural (corn/rye)
Merrit Island (FL)	4[d]	1.8	201	Paola sand	Spodic Quartzipsamment	Scrub-oak/palmetto forest

[a] Organic matter.
[b] Total Kjheldal nitrogen.
[c] pH_w (1:2, w/w).
[d] pH_w (1:1, w/w).

Center for CLPP-BDOBS analyses (described below). Nitrate-N (N_{-NO_3}) and ammonium-N (N_{-NH_4}) in 2 M KCl extracts were analyzed by flow injection analysis on a Lachat Instruments auto-analyzer (Loveland, CO; Hofer, 2003; Knepel, 2003).

2.2. Measurement of cell growth during the assay

Three soils with a range of C and N contents were selected for testing (Table 1). Soils were collected from triplicate plots at Brookings and from the following two sites: a row-crop field soil located at the University of Florida Dairy Research Unit, Hague, FL (29.78° N; 82.42° W) and a scrub-oak forest soil from the National Wildlife Refuge in Merritt Island, FL (28.63° N; 80.7° W). Ten soil cores were taken from the surface layer (0–15 cm depth) and pooled to obtain a composite sample per plot at each site. Samples were shipped in ice and kept at 4 °C until analysis. Complete descriptions of these sites have been reported previously (Johnson et al., 2001; Garland et al., 2010).

2.3. BDOBS plate preparation, inoculation, and reading

The 96-well BDOBS plates (BD Biosciences, Bedford, MA, USA) were used to analyze soils. Plates were pre-filled with 40 μL per well of C solution to determine substrate-induced respiration or of sterile distilled water (SDW) to assess background C use. Wells were also pre-filled with 40 μL of either, a N solution or SDW, to assess N effects on respiration. All the stock solutions and deionized water were filter-sterilized (<0.22 μm, Corning Inc., Lowell, MA, USA) and stored at 4 °C before loading the plates. Soil suspensions were prepared by mixing 10 g fresh soil with 25 mL SDW in a 50 mL BD Falcon centrifuge tube containing ~5 mL sterile glass beads (∅2 mm), and vigorously shaken by hand for 1 min. Slurry (160 μL) was pipetted to each well, resulting in a final well volume of 240 μL. Stock solutions of C (300 mg L^{-1}) and N [60 mg L^{-1} $(NH_4)_2SO_4$] were prepared in order to deliver a final concentration of 50 mg total C substrate L^{-1} and 10 mg N source L^{-1}, respectively. Carbon sources selected for the CLPP analysis were L-arginine, L-asparagine, D-mannose, D-fructose, sodium acetate, propionic acid, vanillic acid and p-coumaric acid (Sigma, St. Louis, MO, USA).

Due to the large volume of soil slurry required for DNA extraction (see below), a custom-made 24-well plate configuration (BD Biosciences, Bedford, MA, USA) was used to study the enrichment effect of substrates and N on microbial growth. Wells were loaded with 0.5 mL^{-1} of stock solutions of C substrates (acetate, mannose, or asparagine [300 mg L^{-1}]) or SDW, and 0.5 mL of stock solutions of $(NH_4)_2SO_4$ [60 mg L^{-1}] or SDW (a no C control). Slurries were prepared as described above, before loading 2 mL $well^{-1}$ of soil suspension. Three replicate soil samples were incubated per plate. Assuming homogeneous mixing of soil suspensions loaded in the wells, added C and N concentrations were 31.25 μg and 6.25 μg g^{-1} soil, respectively. All plates were sealed with Titer Tops film and incubated at 30 °C. Kinetic fluorescence readings were obtained every 15 min for up to 48 h (~16 h in the case of 24-well plates), using a Synergy HT microplate reader (Bio-Tek Instruments, VT, USA), equipped with a 485 nm wavelength excitation filter and a 590 nm wavelength emission filter.

2.4. Fluorescence data analysis

The kinetic fluorescence data were reported as normalized relative fluorescence units (NRFU) by dividing the readings at each time point by the response at 1 h. The fluorescence response shows a peak (i.e., transient minimum in gel dissolved oxygen (DO) concentration) as respiratory activity declines due to exhaustion of the readily available substrate, and as continuing O_2 diffusion regenerates the gel. This peak fluorescent response (F_{max}) was calculated for all samples as an indicator of respiratory activity. Assuming a constant diffusion rate, the minimum DO concentration should reflect the rate of oxygen consumption. The difference in oxygen consumption measured at F_{max} between N-amended and unamended wells, for a given C source (or N_{diff}), was calculated for all substrates tested in 96-well plates. Time-course fluorescence in the 24-well study was plotted and the integrated area under the curve (AUC) was calculated using SigmaPlot (Systat Software, Inc., San Jose, CA, USA).

2.5. Molecular analysis

Two-ml aliquots of the fresh soil slurries were stored at −20 °C before initiating incubation ($T = 0$ samples). At the end of incubation, soil slurries in the 96-well plates were homogenized and a 625 μL-aliquot was transferred from each well to a Bead tube of the Fecal DNA Isolation kit (MoBio, Solana Beach, CA, USA) for DNA extraction. DNA was quantified with Nanodrop ND-1000 (Thermo Fischer Scientific, Inc., Wilmington, DE, USA).

The primer set used was Eub338F/Eub518R for bacteria (Fierer et al., 2005). Each 20 μL reaction mixture contained the following: 10 μL PCR mix Light Cycler 480 SYBR Green I Master Mix (2×, Roche Applied Science, Manheim, Germany), 0.25 μL of each primers (10 μM; Invitrogen); 0.5 μL bovine serum albumina (20 mg ml^{-1}; Roche) and 4 μL PCR-grade ultrapure water, and 5 μL template DNA. Negative (PCR-grade water) and positive DNA control (*Pseudomonas aeruginosa* 10-fold serially diluted) were also included. Reaction conditions were as follows: pre-incubation (95 °C, 10 min, 1 cycle), amplification (95 °C 15 s, 53 °C 15 s, 72 °C 15 s, 45 cycles), in Light Cycler 480 (Roche Applied Science,

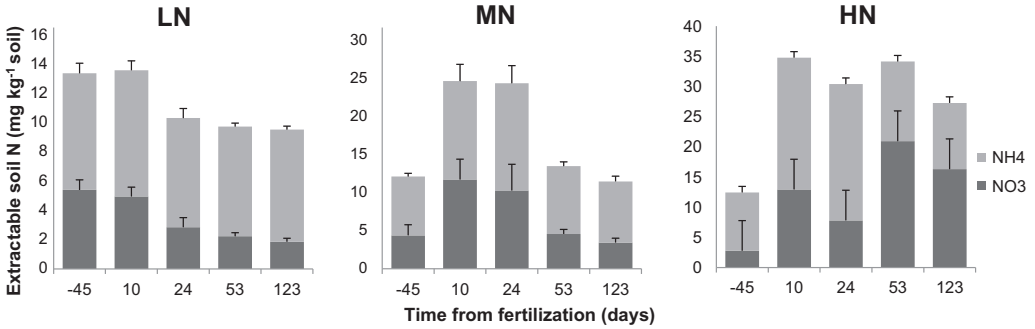

Fig. 1. Extractable soil inorganic nitrogen from the South Dakota test site. All plots received a starter fertilizer (N as urea, P, K) supplying 15.7 kg N ha^{-1} at the time of seeding, 45 days prior to bulk N fertilization with urea. Data represent mean and standard error of samples from three replicate plots receiving no additional N fertilizer (Low Nitrogen, LN); 65 kg ha^{-1} (Medium Nitrogen, MN); or 125 kg ha^{-1} (High Nitrogen, HN) of N fertilizer supplied as urea.

Indianapolis, IN, USA). Copy numbers of small subunit ribosomal RNA (SSU rRNA) genes were calculated from standard curves. Results were expressed as relative abundance; the ratio between the measured copy number in a given substrate/N combination and the initial copy number for that soil.

2.6. Statistical analysis

Statistical analyses of the N_{diff} data were performed as repeated measures data (RM-ANOVA) using R v.2.8.1 (R Development Core Team, 2008). Data were analyzed with a linear mixed-effects model (*lme* procedure fit with REML) using the *nlme* package version 3.1-90 (Pinheiro et al., 2008), to test for the effects of fertilization treatments (F) and time elapsed from fertilization (T_f) on the magnitude of N_{diff}. The overall mixed model included F, T_f, the interaction term $T_f \times F$ (fixed factors) and blocks (random) as the within-subject term. An autoregression with heterogeneous variances model was selected, using Aikake's Information criterion (AIC), Bayesian information criterion (BIC) and log of restricted maximum likelihood (logLik) criterion. We used the Tukey HSD test for multiple comparisons within each day, with a significance of $P < 0.05$.

Relative abundance of bacterial ribosomal gene copy number (RC_B) estimated from qPCR were analyzed with two-tailed, two-sample Student's t-test ($\alpha = 0.05$).

3. Results

3.1. Temporal trends in extractable soil inorganic nitrogen in the field

Field soils displayed differences in soil inorganic N (N_i) concentrations consistent with the fertilization treatments (Fig. 1). The low N (LN) soils contained N_{NH_4} and N_{NO_3} concentrations consistently below 10 mg kg^{-1} both before and after starter fertilizer application, with a slight decrease observed in the mid-point of plant growth 53 days from fertilization. In soil receiving the moderate fertilizer rate (MN), the N_i concentrations increased slightly above 10 mg kg^{-1} at 10 and 24 days following fertilization, and then decreased to similarly low values, comparable to the unfertilized soils. The high N (HN) fertilizer rate caused a transient increase in soil N_{NH_4} of 25 mg kg^{-1} within 24 days from fertilization.

3.2. Temporal trends/field treatment effects in N_{diff}

There were highly significant effects of time elapsed since fertilization on N_{diff} for all C sources and for basal respiration (Table 2). Furthermore, a significant interaction between T_f and F was found for six C sources and the no C control, reflecting different time trends among fertilization treatments (Fig. 2).

In the case of acetate, N_{diff} decreased significantly post-fertilization (10 and 24 d) in all treatments. A marginal interaction (RM-ANOVA $P < 0.1$) is explained by steeper negative slopes (decrease) for HN and MN compared to LN from pre-fertilization and 10 d after fertilization ($P < 0.05$). For mannose, there was a significant interaction (RM-ANOVA $P < 0.001$), as N_{diff} in the LN

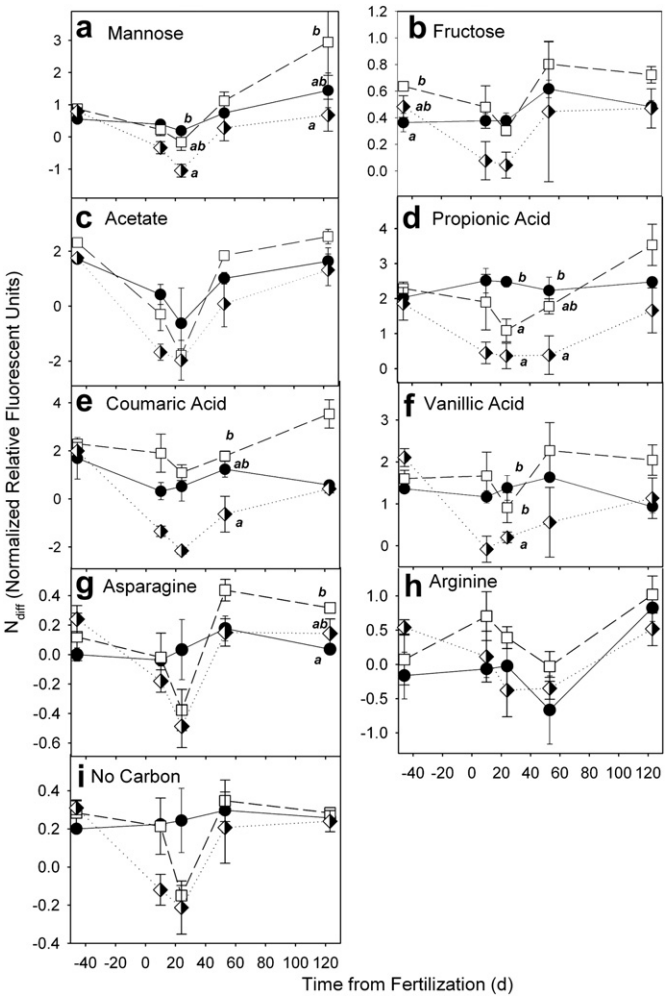

Fig. 2. Temporal response of N_{diff} to different levels of fertilization in Brookings agricultural soil. Data represent mean and standard error of samples from four replicate plots, with responses to different substrates shown in separate panels. Lowercase letters represent significant differences among treatments within a sampling date (HSD, $P < 0.05$). Different lines represent response in soils receiving 15.7 kg ha^{-1} (Low Nitrogen), 65 kg ha^{-1} (Medium Nitrogen), or 125 kg ha^{-1} (High Nitrogen) of N fertilizer supplied as urea. LN = filled circles; MN = open squares; HN = half diamonds.

treatment did not change over time (slope not significant), while N_{diff} for HN decreased post-fertilization (10 and 24 d) compared to LN ($P < 0.01$). N_{diff} for HN was significantly lower than for LN 24 d after fertilization (Tukey HSD, $P < 0.05$). Identical time response was observed with no C added, fructose, asparagine, coumaric acid, and vanillic acid. In the latter, N_{diff} for HN was lower than for LN and MN 24 d after fertilization (Tukey HSD, $P < 0.05$). Propionic acid showed the same time response as described above, except that the differences between HN and LN slopes persisted until 53 d after fertilization. N_{diff} for HN and MN was lower than for LN 24 and 53 d after fertilization (Tukey HSD, $P < 0.05$). Asparagine differed from the other amino acid's time response as N_{diff} increased (positive slope) post-fertilization (123 d after fertilization) in all treatments compared to pre-fertilization status ($P < 0.05$).

Table 2
Results of *RM-ANOVA* for N_{diff} response of Brookings soil, throughout the corn crop cycle. MSE term d.f. = 28.

Effects	Acetate	Propionic acid	Coumaric acid	Vanillic acid	Asparagine	Arginine	Fructose	Mannose	No C
Fertiliz.	$P < 0.001$	$P < 0.001$	$P < 0.01$	$P < 0.001$	$P < 0.01$	*n.s*	$P < 0.001$	$P < 0.01$	*n.s*
T_f	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
$F \times T_f$	*n.s*	$P < 0.05$	$P < 0.01$	$P < 0.001$	$P < 0.05$	n.s	$P < 0.05$	$P < 0.001$	$P < 0.05$

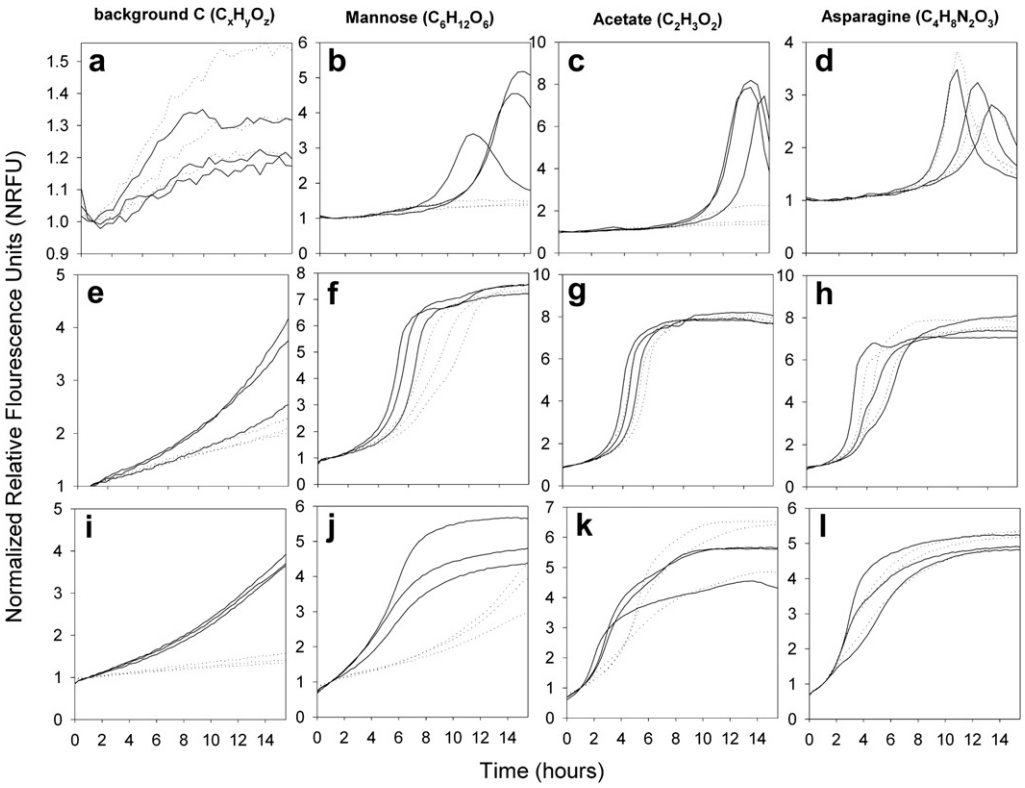

Fig. 3. Time course of fluorescence in 24-well plates used for qPCR measurements. Separate panels show data for different C source amendments (as noted, background C is without any C amendment) for Merritt Island soil (a–d), Hague soil (e–h); and Brookings soil (i–l). Solids lines are replicate wells with N amendment, dotted lines are replicate wells without N amendment. In brackets, chemical formula of the substrates is shown for comparison.

3.3. Measurement of cell growth during the assay

The different soils showed distinctive responses to both C and N amendment (Fig. 3). The scrub soil from Merrit Island had limited fluorescence without C amendment (Fig. 3a), most likely due to the low concentration of available C in this sandy soil. Carbon amendment resulted in a detectable response, but only when N was added (either as inorganic N, or in the case of asparagine, as organic N) and after an 8–10 h lag (Fig. 3b–d). The agricultural soils from Hague and Brookings showed a much greater magnitude of response without C amendment (Fig. 3e, h), but a faster (i.e., lag < 4 h) response to C amendment (Fig. 3f–h and j–l). Statistical analysis of the integral of fluorescent response (Table 3) found that N amendment significantly stimulated respiratory response to mannose for all soils. The scrub soil also showed a positive response to N amendment for acetate use, while basal respiration of the SD soil was stimulated by N addition.

Bacterial growth in the wells based on qPCR estimation of relative ribosomal gene copies (RC_B) also varied among the soils (Table 4). In the Merrit Island soil, RC_b increased 2.5–4 fold in responsive wells (i.e., those receiving both C and N amendment). N addition significantly increased RC_b for mannose and acetate amendment, consistent with the stimulation in respiration discussed above. Growth was less in the agricultural soils, with RCb increasing <2 fold in all tests. The effect of N addition was less clear in the agricultural soils. One test (mannose amendment in the agricultural soil from Brookings) showed a positive growth response consistent with the stimulation in respiratory activity. Two other tests in which respiration was positively affected by N amendment (i.e., mannose use by Hague agricultural soil, basal respiration in Brookings agricultural soil) showed slight, but statistically non-significant, increases in RC_b. Finally, in one test (acetate amendment in Hague agricultural soil), N amendment increased RC_b, but had no effect on respiration.

4. Discussion

As shown in previous work, the major effect of N supplementation during the activity assay is an enhanced peak response in the fluorescent signal (i.e., positive N_{diff}) indicative of a lower transient level of O_2 within the flourorophore-gel matrix on the bottom of the plates. The stimulatory effect of N supplementation was observed with basal soil respiration and induced responses to a variety of C-only substrates. A lack of or weak stimulation by N

Table 3
Substrates respiration with and without N amendment in the BDOBS wells (area units = NRFU × h). Mean and standard errors (SE) of AUC are reported for $n = 3$ replicates.

Soil	No C		Mannose		Acetate		Asparagine	
	−N	+N	−N	+N	−N	+N	−N	+N
Merrit Island	0.80 (0.05)	0.75 (0.03)	0.82 (0.02)	1.26*** (0.04)	0.87 (0.07)	1.65*** (0.12)	0.99 (0.05)	1.01 (0.01)
Hague	0.97 (0.02)	1.19 (0.09)	2.54 (0.13)	3.04* (0.08)	3.55 (0.09)	3.71 (0.06)	3.51 (0.11)	3.49 (0.11)
Brookings	0.74 (0.02)	1.29*** (0.02)	1.24 (0.06)	2.21** (0.20)	2.61 (0.24)	2.61 (0.17)	2.45 (0.10)	2.44 (0.14)

*Significance of the Student's *t*-test between −N and +N ($\alpha = 0.05$, d.f. = 4); SE is shown in brackets. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 4
The relative abundances of bacteria (RC_b) grown with combinations of C and N amendments in the BDOBS wells, in three different soils as estimated using the qPCR assay of SSU 16S rRNA copy number. Error bars are the standard errors (SE) of the mean for $n = 3$ replicates.

Soil	No C		Mannose		Acetate		Asparagine	
	$-N$	$+N$	$-N$	$+N$	$-N$	$+N$	$-N$	$+N$
Merrit Island	1.67 (0.1)	1.49 (0.23)	1.07 (0.11)	3.99* (0.14)	0.93 (0.29)	2.48* (0.27)	3.51 (0.3)	4.09 (0.16)
Hague	1.59 (0.31)	1.72 (0.08)	1.94 (0.14)	2.2 (0.14)	1.12 (0.02)	1.56* (0.18)	1.91 (0.3)	2.05 (0.12)
Brookings	0.76 (0.14)	1.15 (0.07)	0.6 (0.13)	1.47* (0.14)	0.83 (0.22)	0.89 (0.17)	1.97 (0.66)	1.02 (0.12)

*Significance of the Student's t-test between $-N$ and $+N$ ($\alpha = 0.05$, d.f. $= 4$); SE is shown in brackets. * $P < 0.05$.

addition was observed when N-containing substrates were tested, as would be predicted from enhanced intracellular N availability resulting from ammonification. Fertilization in the field and the concomitant increased concentrations of soil available N eliminated N-limited microbial respiration, with the duration of the effect being longer in soils exposed to higher fertilization rates in a manner consistent with field concentrations of inorganic N. These data support the conceptual model that N addition allows for a "burst" of respiration when soil is N-limited (Dilly, 2003; Galicia and García-Oliva, 2004; Teklay et al., 2006).

N is most often considered limiting to the biosynthesis of new biomass (i.e., cell growth) (Nordgren, 1992; Dilly, 1999, 2003; Teklay et al., 2006), although N limitation of microbial activity may be possible in the absence of growth if the regulation of catabolic enzyme production is tightly linked to substrate availability (Geisseler and Horwath, 2008). In this study, increased respiratory response with N amendment was typically associated with increased growth. Although for rapidly responding soils (i.e., the agricultural soils), some C sources showed N-limited use without detectable increases in growth, probably due to the exhaustion of the relatively low levels of C amendment before growth could be detected. The scrub soil with a slower respiratory response also showed a much stronger growth response to N, consistent with the idea that the lag period is associated with induction of bacterial growth. The growth observed in these soils amended with N is comparable to the bacteria biomass increase measured by direct counts by Nannipieri et al. (1978) in soils amended with C + N

Overall, these data suggest that the degree of selective enrichment in the assay is relatively minor, particularly for soils with minimal lag in respiratory response. This indicates that the assay reflects how the *in situ* soil community is poised to utilize various sources of C (and N), rather than a reflection of a small fraction of copiotrophic organism response, as in the previous version of the CLPP technique (i.e. based on Biolog plates). This allows for temporal dynamics in the community physiological state to be effectively assessed, as reflected by the changes in N limitation as a function of time from fertilization in the present study. The slight increases in biomass (i.e. <2-fold) may be an effective means of understanding what portion of the community is responding to different stimuli if accompanied by molecular approaches to identify activated organisms in the wells.

No attempt was made to estimate bacterial numbers from copy numbers of ribosomal genes, due to several variables that limit the efficacy of the technique for the analysis of mixed microbial populations in environmental samples: 1) redundancy of rRNA operons and intra-genomic sequence heterogeneity (Klappenbach et al., 2001); 2) variation in operon numbers between species; 3) lack of relation between phylogeny and operon numbers (Klappenbach et al., 2001). Moreover, fractional copy numbers (with respect to time or treatment) have been shown to provide a more accurate index of target abundances, since the efficiency of PCR amplification can vary across DNA samples (Fierer et al., 2005). We attempted to quantify the response of fungi in wells (unreported results) using qPCR of fungal specific primers (Fierer et al., 2005), but had limited success, probably due to the insufficient sample size in the wells (Ranjard et al., 2003). Previous studies have demonstrated that both fungi and bacteria respond in the assay (Väisänen et al., 2005; Zabaloy et al., 2008), so further evaluation of the fungal growth response to N is necessary to fully assess the interaction between community respiration and microbial growth within the assay.

Nitrogen supplementation in the assay reduced the respiratory response toward certain substrates (i.e., negative N_{diff}) in the Brookings agricultural soil. Reduced respiration with supplemental N could be caused by more efficient C assimilation, as has been reported by Dilly (1999). However, we generally observed that N supplementation either increased respiration or had no effect. The negative effect was most pronounced 2–3 weeks after fertilization in the field, particularly at the highest N fertilization rate. Direct toxicity caused by the amendment is unlikely given the low level of addition (i.e., less than 10 $\mu g\, N_{NH_4}$ or S_{-SO_4} g^{-1} soil). While the cause for this inhibitory effect is unclear, it is interesting to note that a similar effect was observed in the agricultural soil from Hague following fertilization with organic wastes (i.e., either dairy waste solids or biosolids), but not mineral fertilizer (Garland et al., 2010). Observation of the effect in the present study in relation to increasing levels of urea application supports the hypothesis that the negative N_{diff} values result from a decrease in energy dependent uptake of organic N forms, such as urea (Jahns et al., 1998), when N_{NH_4} is added to the wells. In this scenario, addition of N_{NH_4} does not inhibit activity, but instead reduces the respiratory demand, thereby decreasing the rate of O_2 consumption. Further work comparing soils receiving organic and inorganic N fertilization are needed to determine if negative N_{diff} values reflect preferential use of organic N by the soil microbial community. Concurrent measurement of yield efficiency (e.g, assimilation versus respiration of radiolabelled isotopes) would also be useful for defining the mechanistic basis for negative N_{diff}.

The Brookings agricultural soil responded to different substrates inconsistently, even when comparing between two members of the same class of compounds (e.g., fatty acids, monosaccharides, and phenolics). Use of propionic acid, for example, was more consistently N-limited across time and among fertilization treatments, than it was for acetate. N_{diff} decreased to negative values for mannose, acetate, and coumaric acid, following fertilization, but it did not for fructose, propionic acid, and vanilic acid. Substrate-specific variation in N_{diff} for a given soil sample suggests that the assay may reflect differences in the degree of N limitation amongst various components of the microbial community. Linking the CLPP-BDOBS with molecular profiling of the wells could provide insight into the specific nature of this variability.

5. Conclusions

Field experiments using a microplate-based O_2 sensor confirmed preliminary evidence that the difference in soil microbial respiration with and without N amendment to the assay (i.e.,

N_{diff}) reflects the N availability in the soil. N_{diff} decreased in response to urea fertilization in the field and the extent and duration of the reduction was greater at higher levels of fertilization. N_{diff} varied among different types of C amendments in a given soil sample, suggesting a complexity in N limitation within different components of the soil microbial community worthy of future study. Testing three different soil types indicated that the increase in respiration was usually, but not always, associated with enhanced bacterial growth in the assay. The relatively limited amount of growth (1 to 4-fold) demonstrates that the method is a rapid means of effectively assessing the physiological capabilities of soil microbial communities.

Acknowledgements

The Brookings (SD) field plots were managed by Dr. Joe Pikul with assistance from USDA-ARS employees Max Pravacek and Dave Harris. Assistance in the lab and field was provided by USDA-ARS employees Amy Christie, Levi Vanoverschelde, Anne Weinkauf, and Dynamac employee John Catechis.

Role of the funding source

This work was funded through a grant from the Soil Processes Program of the Cooperative State Research, Education, and Extension Service (CSREES), United States Department of Agriculture (USDA). The National University of the South (UNS, Argentina) funded M.C. Zabaloy's internship at the Space Life Sciences Laboratory (Kennedy Space Center). None of the funding sources was involved in the executive work or the writing of the manuscript.

References

Brown, A., Garland, J.L., Day, F.P., 2009. Physiological profiling of soil microbial communities in a Florida scrub-oak ecosystem: spatial distribution and nutrient limitations. Microbial Ecology 57, 14–24.

Demoling, F., Figueroa, D., Baath, E., 2007. Comparison of factors limiting bacterial growth in different soils. Soil Biology & Biochemistry 39, 2465–2495.

De Nobili, M., Contin, M., Mondini, C., Brookes, P.C., 2001. Soil microbial biomass is triggered by trace amounts of substrates. Soil Biology & Biochemistry 9, 1163–1170.

Dilly, O., 1999. Nitrogen and phosphorus requirement of the microbiota in soils of the Bornhöved Lake district. Plant and Soil 212, 175–183.

Dilly, O., 2003. Regulation of the respiratory quotient of soil microbiota by availability of nutrients. FEMS Microbiology Ecology 43, 375–381.

Fierer, N., Jackson, J.A., Vilgays, R., Jackson, R.B., 2005. Assessment of soil microbial community structure by use of taxon – specific quantitative PCR assays. Applied and Environmental Microbiology 71, 4117–4120.

Galicia, L., García-Oliva, F., 2004. The effects of C, N and P additions on soil microbial activity under two remanent tree species in a tropical seasonal pasture. Applied Soil Ecology 26, 31–39.

Garland, J.L., Mills, A.I., 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Applied and Environmental Microbiology 57, 2351–2359.

Garland, J.L., Roberts, M.S., Levine, L.H., Mills, A.L., 2003. Community-level physiological profiling using an oxygen-sensitive fluorophore in a microtiter plate. Applied and Environmental Microbiology 69, 2994–2998.

Garland, J.L., Mackowiak, C.L., Zabaloy, M.C., 2010. Organic waste amendment effects on soil microbial activity in a corn–rye rotation: application of a new approach to community-level physiological profiling. Applied Soil Ecology 44, 262–269.

Geisseler, D., Horwath, W.R., 2008. Regulation of extracellular protease activity in soil in response to different sources and concentrations of nitrogen and carbon. Soil Biology & Biochemistry 40, 3040–3048.

Gnankambary, Z., Lstedt, U., Nyberg, G., Hien, V., Malmer, A., 2008. Nitrogen and phosphorus limitation of soil microbial respiration in two tropical agroforestry parklands in the south-Sudanese zone of Burkina Faso: the effects of tree canopy and fertilization. Soil Biology & Biochemistry 40, 350–359.

Hofer, S., 2003. Determination of Ammonia (Salicylate) in 2M KCl soil extracts by Flow Injection Analysis. QuikChem Method 12-107-06-2-A.

Jahns, T., Zobel, A., Kleiner, D., Kaltwasser, H., 1998. Evidence for carrier-mediated, energy-dependent uptake of urea in some bacteria. Archives of Microbiology 149, 377–383.

Johnson, D.W., Hungate, B.A., Dijkstra, P., Hymus, G., Drake, B., 2001. Effects of elevated carbon dioxide on soils in a Florida scrub oak ecosystem. Journal of Environmental Quality 30, 501–507.

Klappenbach, J.A., Saxman, P.R., Cole, J.R., Schmidt, T.M., 2001. rrndb: The ribosomal RNA operon copy number data base. Nucleic Acids Research 29, 181–184.

Knepel, K., 2003. Determination of nitrate in 2M KCl soil extracts by flow injection analysis. QuikChem Method 12-107-04-1-B.

Liu, Y., 1998. Energy uncoupling in microbial growth under substrate-sufficient conditions. Applied Microbiology & Biotechnology 49, 500–505.

Manzoni, S., Jackson, R.B., Trofymow, J.A., Porporato, A., 2008. The global stoichiometry of litter nitrogen minerlization. Science 321, 684–686.

Nannipieri, P., Johnson, R.L., Paul, E.A., 1978. Criteria for measurement of microbial growth and activity in soil. Soil Biology & Biochemistry 10, 223–229.

Nordgren, A., 1992. A method for determining microbially available N and P in an organic soil. Biology & Fertility of Soils 13, 195–199.

Pikul, J.L., Schumacher, T.E., Vigil, M., 2001. Nitrogen use and carbon sequestered by corn rotations in the northern corn belt, U.S. The Scientific World 1, 707–713.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., 2008. nlme: Linear and nonlinear mixed effects models. R package version 3.1-90.

R Development Core Team, 2008. R: A Language and Environment for Statistical Computing. v.2.8.1. The R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0.

Ranjard, L., Lejon, D.P.H., Mougel, C., Schehrer, L., Merdinoglu, D., Chaussod, R., 2003. Sampling strategy in molecular microbial ecology: influence of soil sample size on DNA fingerprinting analysis of fungal and bacterial communities. Environmental Microbiology 5 (11), 1111–1120.

Rinnan, R., Baath, E., 2009. Differential utilization of carbon substrates by bacteria and fungi in tundra soil. Applied and Environmental Microbiology 75, 3611–3620.

Russell, J.B., Cook, G.M., 1995. Energetics of bacterial growth: balance of anabolic and catabolic reactions. Microbiological Reviews 59, 48–62.

Schimel, J.P., Weintraub, M.N., 2003. The implications of exoenzyme activity on microbial carbon and nitrogen limitation in soil: a theoretical model. Soil Biology & Biochemistry 35, 549–563.

Teixeira de Mattos, M.J., Neijessel, O.M., 1997. Bioenergetic consequences of microbial adaptation to low-nutrient environments. Journal of Biotechnology 59, 117–126.

Teklay, T., Nordgren, A., Malmer, A., 2006. Soil respiration characteristics of tropical soils from agricultural and forestry land-uses at Wondo Genet (Ethiopia) in response to C, N and P amendments. Soil Biology & Biochemistry 38, 125–133.

Tempest, D.W., 1978. Eco-physiological aspects of microbial growth in aerobic nutrient-limited environments. Advances in Microbial Ecology 2, 105–153.

Thiet, R.K., Frey, S.D., Six, J., 2006. Growth yield efficiencies of soil microbial communities with different fungal:bacterial ratios. Soil Biology & Biochemistry 38, 837–844.

Väisänen, R.K., Roberts, M.S., Garland, J.L., Frey, S.D., Dawson, L.A., 2005. Physiological and molecular characterization of microbial communities associated with different water-stable aggregate size fractions. Soil Biology & Biochemistry 37, 2007–2016.

Zabaloy, M.C., Lehman, R.M., Frey, S.D., Garland, J.L., 2008. Optimization of an oxygen-based approach for community-level physiological profiling of soils. Soil Biology & Biochemistry 40, 2960–2969.