Biol Fertil Soils (2004) 40: 201–205
DOI 10.1007/s00374-004-0760-4

SHORT COMMUNICATION

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A microplate assay to measure soil microbial biomass phosphorus

Received: 7 October 2003 / Revised: 17 February 2004 / Accepted: 17 February 2004 / Published online: 20 May 2004

Abstract Quantification of phosphorus (P) concentrations in microbial biomass is required to better understand how P immobilization and turnover in soils are controlled by environmental and anthropogenic factors. Soil microbial biomass P (MBP) is generally extracted using the chloroform fumigation-direct extraction procedure and then analysed for P using the ammonium molybdate-ascorbic acid method on a flow injection analysis (FIA) system. Our objective was to determine whether a microscale malachite green method on a microplate system would provide as accurate MBP analysis as the ascorbic acid method on an FIA system. Twelve soils were collected from agricultural fields in southwestern Quebec, fumigated with chloroform and extracted with 0.5 M $NaHCO_3$ (pH 8.5). The dissolved inorganic phosphorus (DIP) concentration in fumigated soils was not affected by the method of analysis, and results from the two systems of analysis were significantly correlated (r =0.998, P <0.05). The MBP concentrations in these agricultural soils were between 0.36 and 60.05 μg P g^{-1}, consistent with other published values. Our results indicate that MBP can be assessed equally well with the malachite green method using a microplate system as with the ascorbic acid method on an FIA system. The microplate system is rapid and requires smaller volumes of samples and reagents than the FIA system, thus reducing the quantity of waste produced. We conclude that the microscale malachite green method could be applied to measure the MBP concentration in a wide range of soils with good sensitivity, reproducibility and accuracy.

Keywords Malachite green · Ascorbic acid · Soil microbial biomass · Soil phosphorus analysis · Microscale assay

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Introduction

The fundamental role of soil microorganisms in processes such as decomposition, nutrient cycling, aggregation and plant growth is influenced by the biomass and activity of soil microbial communities. Soil microbial biomass is often measured using chloroform fumigation-direct extraction, which permits quantification of the carbon, nitrogen and phosphorus concentrations in the microbial biomass (Vance et al. 1987; Dalal 1998). Although soil microbial biomass C and N levels are reported routinely, fewer studies provide estimates of microbial biomass phosphorus (MBP). Information on MBP pool dynamics is required to better understand how P immobilization and turnover in soils are controlled by environmental (e.g. climate, soil type, topography) and anthropogenic (e.g. fertilizers, pesticides, crops, tillage) factors (He et al. 2003).

The MBP concentration is generally assessed with the ammonium molybdate-ascorbic acid method of Murphy and Riley (1962). However, the ammonium molybdate-malachite green method (Petitou et al. 1978) may be more accurate at submicromolar P concentrations. The malachite green method had a lower detection limit for P in Olsen-P, calcium chloride and water extracts than the ascorbic acid method (Ohno and Zibilske 1991; Subba Rao et al. 1997). Originally designed for manual spectroscopy determination, these colorimetric methods were later adapted for FIA systems, thus increasing the sensitivity and repeatability of measurements (Novozamsky et al. 1993; Coventry et al. 2001). Recently, D'Angelo et al. (2001) adapted the malachite green method to a 96-

well microtiter plate format for P analysis in water, KCl, $CaCl_2$, NaOH and HCl soil extracts. Miniaturization of this colorimetric assay to the microplate scale decreases the volume of chemical reagents used and wastes produced, increases throughput and reduces the need for the expensive equipment and technical expertise required to run, for example, an FIA system. While the microscale malachite green method may be useful for rapid, sensitive analysis of MBP, it is necessary to verify that the chloroform fumigation-direct extraction procedure does not produce compounds that interfere with P detection.

The objective of this study was to determine the suitability of using the malachite green method for MBP analysis, and to compare results from a microplate system with an FIA system.

Materials and methods

Soil collection, storage and characterization

We collected soils (mixed, frigid Typic Endoaquents) from the top 15 cm of agricultural fields in southwestern Quebec, Canada in 1999 (soils 1–8) and 2001 (soils 9–12). These soils came from fields under soybean (*Glycine max* L.), bean (*Phaseolus vulgaris* L.) and asparagus (*Asparagus officinalis* L.) production, and were selected based on their wide range of physical and chemical properties (Table 1). After collection, half of each soil sample was air-dried, sieved (<2-mm mesh) and stored at room temperature for soil physical and chemical analysis, while the other half was frozen immediately and stored at −20°C until MBP analysis was conducted.

Extraction and determination of MBP with microplate and FIA systems

All extractions were done in triplicate for each soil and soil weights are expressed on an oven-dry basis. Frozen soil samples were defrosted, and about 2 g soil was immediately shaken with 40 ml 0.5 M $NaHCO_3$ (pH 8.5) for 30 min and filtered through Whatman 42 (Olsen et al. 1954). A second soil sample (2.5 g defrosted soil) was spiked with 250 µl of 250 mg P l^{-1} (from KH_2PO_4) to deliver 25 µg P g^{-1} immediately before the soil was extracted with 0.5 M $NaHCO_3$ (pH 8.5) solution. Approximately 2 g defrosted soil was fumigated with chloroform for 24 h (Brookes et al. 1982) and then extracted with $NaHCO_3$ following the procedure above. A 1-ml aliquot of each $NaHCO_3$ extract was neutralized with 0.375 ml 10% H_2SO_4, vortexed and diluted with 10 ml double-distilled water prior to analysis (Subba Rao et al. 1997). The microbial biomass P (MBP) concentration (µg P g^{-1} soil) was calculated as

$$\mathrm{MBP} = \frac{\left(\mathrm{DIP}_{\mathrm{fumigated}} - \mathrm{DIP}_{\mathrm{unfumigated}}\right)}{\left(K_{EP} \times \%\ \mathrm{recovery}\right)} \quad (1)$$

where $DIP_{fumigated}$ is the dissolved inorganic P (DIP) concentration (µg P g^{-1} soil) in $NaHCO_3$ extracts of fumigated soil, $DIP_{unfumigated}$ is the DIP concentration (µg P g^{-1} soil) in $NaHCO_3$ extracts of unfumigated soil, K_{EP} is 0.4, accounting for the efficiency of P extraction from lysed microbial cells (Brookes et al. 1982; Hedley and Stewart 1982), and % recovery is the proportion of spike recovered in each unfumigated soil sample, as calculated from Eq. 2.

$$\%\ \mathrm{recovery} = \frac{100\% \times \left(\mathrm{DIP}_{\mathrm{spiked}} \times V_{t-sp}\right)}{\left[\left(\mathrm{DIP}_{\mathrm{unspiked}} \times V_{t-uf}\right) + \left(C_{\mathrm{spike}} V_{\mathrm{spike}}\right)\right]} \quad (2)$$

where DIP_{spike} is the DIP concentration of spiked soil (mg P l^{-1}), $V_{t\text{-}sp}$ is the soil solution volume (l) in spiked soils, $DIP_{unspiked}$ is the DIP concentration of unfumigated, unspiked soil (mg P l^{-1}), $V_{t\text{-}uf}$ is the soil solution volume (l) in unfumigated soils, C_{spike} is the concentration of spike solution (in this experiment, 250 mg P l^{-1}),

Table 1 Selected physical and chemical properties of soil (0–15 cm) materials used in the study

Soil[a]	Crop	pH[b]	OM[c] (g kg^{-1})	Clay[d] (g kg^{-1})	Silt[d] (g kg^{-1})	Sand[d] (g kg^{-1})	Textural class	Olsen-P[f]		
								Mehlich-III P[e] (mg kg^{-1})	Ascorbic acid (FIA) (mg kg^{-1})	Malachite green (microplate) (mg kg^{-1})
1	Soybean	6.9	51	545	360	95	Clay	7.8	9.0	8.82
2	Soybean	7.0	53	383	588	29	Silty clay loam	193.6	89.8	91.0
3	Soybean	6.7	27	295	566	139	Silty clay loam	50.0	32.0	30.1
4	Bean	5.4	51	380	310	310	Clay loam	29.7	15.9	14.6
5	Bean	6.5	36	258	473	269	Loam	183.0	86.3	87.4
6	Bean	5.0	24	343	429	228	Clay loam	125.0	61.4	59.9
7	Bean	5.3	41	308	327	365	Clay loam	170.6	94.8	99.2
8	Soybean	6.4	31	392	508	100	Silty clay loam	34.6	21.3	22.0
9	Asparagus	5.9	88	50	200	750	Sandy loam	579.0	93.7	95.9
10	Asparagus	6.5	27	30	90	880	Sand	569.0	68.8	63.1
11	Asparagus	7.1	107	50	290	660	Sandy loam	175.0	56.0	61.8
12	Asparagus	6.4	117	100	350	550	Sandy loam	236.0	81.6	72.5

[a] All soils are Typic Endoquents
[b] Soil/water extracts (1:2 soil/solution ratio; Hendershot et al. 1993)
[c] Organic Matter (*OM*) determined by loss on ignition (360°C for 4 h; Schulte et al. 1991)
[d] Particle-size analysis (Sheldrick and Wang 1993)
[e] Soil/Mehlich-III extractable P (1:5 soil/solution ratio) analysed with a Lachat-FIA system (Sen Tran and Simard 1993)
[f] Soil:0.5 M $NaHCO_3$ (pH 8.5) extracts (1:20 soil/solution ratio; Olsen et al. 1954) analysed with the ascorbic acid method on a Lachat-FIA system and the malachite green method with a microplate system

and V_{spike} is the volume of spike added (in this experiment, 0.00025 l).

The DIP concentration in unfumigated and fumigated $NaHCO_3$ extracts was measured on a microplate system using the ammonium molybdate-malachite green assay developed by D'Angelo et al. (2001). Briefly, 200 µl diluted and neutralized $NaHCO_3$ extract was mixed with 40 µl Reagent 1 (14.2 mmol l^{-1} ammonium molybdate tetrahydrate in 3.1 M H_2SO_4) in disposable 96-well polystyrene microplates (Becton Dickinson Labware, Franklin Lakes, N.J.) for 10 min on an orbital shaker. Then, 40 µl of Reagent 2 was added and the plate was shaken more rapidly for an additional 20 min. Reagent 2 contained 3.5 g l^{-1} aqueous polyvinyl alcohol reagent containing 0.35 g l^{-1} malachite green carbinol hydrochloride (Sigma, St Louis, Mo.). Both reagents are stable at room temperature and the colour produced by ammonium molybdate-malachite green reaction was stable for at least 24 h (data not shown). Absorbance was measured at 600 nm using a Bio-Tek Model EL 309 microplate reader (Bio-Tek Instruments, Winooski, Vt.). The DIP concentration in fumigated and unfumigated $NaHCO_3$ extracts was also measured on an FIA system using the ammonium molybdate-ascorbic acid procedure of Murphy and Riley (1962) with a Lachat Quik-Chem AE flow-injection autoanalyzer (Lachat Instruments, Milwaukee, Wis.).

Statistical analysis

The values presented are means (± standard errors). All results are given on an oven-dry soil basis. Analysis of variance, correlation and linear regression were performed using CoStat, version 6.003 (CoHort Software, Monterey, Calif.).

Results and discussion

The DIP concentration measured in fumigated soils by the malachite green method on a microplate system ranged from 13.8 to 143.7 µg P g^{-1} soil, and was between 17.6 and 145.0 µg P g^{-1} soil using the ascorbic acid method on an FIA system (Table 2). The results were not affected by the method of analysis (P =0.784), although there was significant (P <0.05) variation between soil samples. Results from these two methods of P analysis were significantly correlated (r =0.998±0.020, P <0.05, n =12). The Olsen-P concentrations measured with the malachite green and ascorbic acid methods (Table 1) were also significantly correlated (r =0.992±0.039, P <0.05, n =12), which is comparable to the relationship described by Subba Rao et al. (1997) for Olsen-P analysed with these methods (r =0.975±0.079, P <0.05, n =10).

The analytical characteristics of the microplate and FIA systems are presented in Table 3. The FIA system had a higher limit of determination (2 mg P l^{-1}) and a lower detection limit (0.006 mg P l^{-1}) than the microplate system. The minimum detection limit of our microplate system was higher than the detection limit of 0.006 mg P l^{-1} reported by D'Angelo et al. (2001). The relative standard deviation of all standards ranged from 0.44% to 18.73% (n =9) for the FIA system and 1.28% to 5.45% (n =27) for the microplate system, indicating good reproducibility. The sensitivity of analysis, as expressed by the molar absorptivity, was closer to the sensitivity reported by D'Angelo et al. (2001) of 46,841±2,400 M^{-1} cm^{-1} than the sensitivity of 64,641±743 M^{-1} cm^{-1} observed by Ohno and Zibilske (1991). Our results indicate that P analysis with the malachite green method in a microplate system is comparable in accuracy to P results obtained with the ascorbic acid method on an FIA system.

Soils were stored in a freezer for up to 2 years before analysis, which was not optimal for assessing the MBP concentration and probably contributed to low values of MBP in some samples, such as soils 1 and 10 (Table 4). The MBP concentrations were between 0.36 and 60.05 µg P g^{-1} (Table 4), and were in the range of published values. The MBP pool in British agricultural soils contained 5.3–106.0 µg P g^{-1} (Brookes et al. 1984) while pasture soils in England and New Zealand had MBP concentrations between 20 and 239 µg P g^{-1} (Perrott and Sarathchandra 1989; Turner and Haygarth 2003). The recovery of P from samples spiked with 25 µg P g^{-1} soil ranged from 70.9% to 118.8% (Table 4), and was assessed to correct for P sorption by soils during the chloroform fumigation-direct extraction processes (Brookes et al. 1982). The P adsorption capacity is affected by factors such as soil pH, texture, and the degree of P saturation in the soil matrix (Bache and Williams 1971). In other studies, the recovery of P from spiked samples ranged from 20.8% to 99.6% (Brookes et al. 1982; Perrott and Sarathchandra 1989).

Our results indicate that MBP can be assessed equally well with the malachite green method using a microplate system as with the ascorbic acid method on an FIA

Table 2 The dissolved inorganic P (DIP) concentration (± standard error, n =3) in $NaHCO_3$ extracts of chloroform-fumigated soil samples analysed by the ascorbic acid method on an FIA system and the malachite green method on a microplate system

Soil	DIP (ascorbic acid method) (µg P g^{-1} soil)	DIP (malachite green method) (µg P g^{-1} soil)
1	17.6 (2.0)	13.8 (2.2)
2	78.5 (0.5)	81.3 (1.9)
3	33.3 (0.3)	34.5 (0.5)
4	21.0 (0.4)	19.3 (0.7)
5	98.3 (1.5)	93.8 (1.7)
6	75.1 (1.8)	74.2 (2.5)
7	145.0 (11.4)	143.7 (9.7)
8	40.2 (2.1)	39.7 (4.2)
9	125.0 (3.4)	121.8 (7.2)
10	96.5 (0.6)	99.6 (1.4)
11	71.5 (0.3)	73.1 (0.8)
12	87.6 (1.6)	89.5 (2.5)

Table 3 Analytical characteristics of the two methods of P determination in soil extracts used in this study

Parameter	Ammonium molybdate-ascorbic acid method (Lachat flow injection analysis system)[a]	Ammonium molybdate-malachite green method (microplate system)[b]
Correlation (*r*)	0.999	0.998
Standard deviation of *r*	0.000	0.001
Probability of *r* =0	*P* <0.0001	*P* <0.0001
n[c]	6	5
Slope (*b*)	7.643×10^{6}	1.636
Y intercept (*a*)	-2.806×10^{4}	0.092
Standard deviation of *b*	3.952×10^{4}	0.062
Molar absorptivity	$2.367\times10^{11}\pm1.224\times10^{9}$ μV-sM^{-1} cm^{-1}	50,662.1±1,924.1 OD units M^{-1} cm^{-1}
Limit of determination (mg P l^{-1})	2.000	1.000
Concentration range (mg P l^{-1})	0.006–2.000	0.007–1.000
Blank measurement	7.920×10^{4}	0.007
Standard deviation of blank	1.484×10^{4}	0.004
Standard error of mean for blank	4.945×10^{3}	0.001
Limit of detection (mg P l^{-1})	0.006	0.007

[a] Standard curves for P determination by the Lachat FIA system was a plot of mg P l^{-1} vs μV s
[b] Standard curves for P determination by the microplate system was a plot of mg P l^{-1} vs optical density at 600 nm
[c] *n* is the number of points in the standard curve used for the determination of analytical parameters for each method (*n* =9 for ascorbic acid method, *n* =27 for malachite green method)

system. The microplate system requires less sample volume than the FIA system, reducing the quantities of reagents needed and wastes produced. The microplate system may increase laboratory efficiency because it does not require dedicated equipment or technical expertise, like the FIA system. A well-organized technician can run more than 200 samples a day. A microplate is read in 30 s and most microplate readers are interfaced with computers, facilitating the calculation of P concentrations from absorbance values. The reagents used for the malachite green method are stable for about 1 year, and the colour developed by this method is stable for approximately 24 h. The malachite green method using a microplate system is already in use for P analysis in water and soil extracts (D'Angelo et al. 2001), and could be applied to measure the MBP concentration in soils.

Acknowledgements This project was supported by the Fonds Formation de Chercheurs et l'Aide à la Recherche (FCAR) and Institut de Recherche et de Développement en Agroenvironnement (IRDA). We thank Dr. Gaetan Faubert for allowing us to use the microplate reader and Hélène Lalande for technical assistance with the Lachat-FIA analysis.

Table 4 The dissolved inorganic P (DIP) concentration (± standard error, *n* =3) in $NaHCO_3$ extracts of unfumigated soil, chloroform fumigated soil and unfumigated soil plus 25 μg P g^{-1} (spiked soil), as measured by the malachite green method on a microplate system

Soil	Unfumigated soil (DIP, μg P g^{-1} soil)	Fumigated soil (DIP, μg P g^{-1} soil)	Spiked soil (DIP, μg P g^{-1} soil)	% Recovery[a]	MBP[b] (μg P g^{-1} soil)
1	13.7 (4.0)	13.8 (2.2)	33.8 (0.4)	74.9	0.5
2	70.6 (3.8)	81.3 (1.9)	96.1 (4.0)	97.3	27.5
3	32.2 (0.8)	34.5 (0.5)	52.0 (2.9)	86.8	6.4
4	14.4 (0.8)	19.3 (0.7)	31.6 (2.1)	70.9	17.4
5	73.5 (1.7)	93.8 (1.7)	114.5 (2.7)	113.7	44.6
6	65.3 (3.0)	74.2 (2.5)	97.2 (3.3)	107.1	20.8
7	115.2 (2.9)	143.8 (9.7)	162.2 (4.5)	118.8	60.0
8	32.1 (7.9)	39.7 (4.2)	58.0 (5.7)	89.7	21.2
9	114.9 (5.2)	121.8 (7.2)	141.0 (4.4)	96.4	17.9
10	99.4 (1.2)	99.6 (1.4)	114.7 (1.8)	88.5	0.4
11	63.4 (1.8)	73.1 (0.8)	87.9 (4.8)	90.9	26.8
12	76.5 (1.7)	89.5 (2.5)	99.3 (8.4)	95.1	34.2

[a] The % recovery was estimated from Eq. 2
[b] Microbial biomass P (MBP) was estimated from Eq. 1

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