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Short communication

Microplate-scale fluorometric soil enzyme assays as tools to assess soil quality in a long-term agricultural field experiment

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ABSTRACT

We investigated the potential of microplate-scale fluorometric soil enzyme assays to differentiate plots under contrasting long-term organic and mineral N fertilization regimens to determine the relevance of this analytical approach to soil quality related studies.

Enzymes involved in the breakdown of cellulose and hemicellulose showed maximum activities in plots amended with manure. Conversely, the enzymes involved in the hydrolysis of starch and phosphate esters peaked under mineral N fertilization. Linear regression analysis indicated close associations between enzyme activities and other fundamental soil properties related to soil quality, and principal component analysis separated the soil samples according to their responses to organic and mineral N fertilization. We conclude that microplate-scale fluorimetry is a fast throughput tool for the measurement of multiple soil enzyme activities as soil quality indicators.

Soil enzymes catalyze a complex web of important reactions for life processes of microorganisms, depolymerization of macromolecular organics, cycling of nutrients, and formation of organic matter and soil structure (Dick, 1994). Changes in enzyme activities provide an early indication of modifications in fundamental soil biochemical processes and thus could represent a useful tool to assess and monitor soil quality (Schloter et al., 2003).

Microplate-scale fluorometric assays based on the use of 4-methylumbelliferone (MUF) offer a fast throughput approach for studying the response of multiple enzyme activities involved in the main soil biochemical processes of organic matter degradation and nutrient cycling. Since Marx et al. (2001) and Vepsäläinen et al. (2001) adapted MUF based enzymatic assays to the microplate scale, this analytical approach has become prevalent in studies of soil enzymes (German et al., 2011). However microplate-scale fluorometric enzyme assays have been tested on only a narrow range of soil types (Trap et al., 2012). We therefore believe that, in order to make full use of enzyme activities estimated by microplate-scale fluorimetry as indicators of soil quality, more information on their responses to different management practices is needed. Further, since the use of soil enzyme activities as soil quality indicators is based on the close relationship of soil enzymes to other important physical, chemical and microbiological properties determining soil quality (Dick, 1994), we wanted to verify whether these relationships are observed as well when enzyme activities are estimated by microplate-scale fluorimetry.

We therefore investigated the potential of microplate-scale fluorometric soil enzyme assays to differentiate agricultural plots under contrasting organic and mineral N fertilization regimens. A long-term experiment was selected to detect variations in slowly changing soil properties, such as soil organic matter content (Kandeler et al., 1999; Körschens, 2006), and to relate these variations to soil enzyme profiles.

In March 2012, surface soil samples (0–20 cm) were collected at a field experiment established in 1966 at the University of Bologna Experimental Farm, in the South-east of the Po valley (Italy, 44°33′ N, 11° 24′ E; 23 m.a.s.l.). The soil was classified as a fine silty, mixed, mesic Udic Ustochrepts (USDA Soil Taxonomy) and contained 56% sand, 16% silt and 28% clay (Triberti et al., 2008). Maize (*Zea mays* L.) and winter wheat (*Triticum aestivum* L.) were cropped in a 2-year rain fed rotation. The experimental design was a split-plot with four replicated blocks. The main factor was organic fertilization and the split factor was mineral N fertilization. Each block was initially divided into main plots that were randomly allocated to the following treatments: un-amended control (C), wheat or maize crop residues (CR), and cattle manure (M). The organic fertilizers were applied every year at the same dry matter rate, corresponding to 6.0 t dry matter ha^{-1} for wheat and to 7.5 t dry matter ha^{-1} for maize. Each plot was then split to receive 0 (N_0) or 200 (N_{200}) kg ha^{-1} of mineral N, supplied as urea.

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Soil was sieved through a 2 mm sieve and thoroughly mixed. Soil water content was determined by drying soil samples to constant mass at 105 °C. Soil pH was measured using a glass electrode in 1:5 (v:v) suspensions of air dried soil in 10 mM $CaCl_2$. Carbonate content was determined according to the Dietrich-Frühling method. Soil organic carbon (SOC) and total nitrogen (TN) content were determined on air dried, finely ground soil aliquots with an elemental analyzer (CHNS-O Elemental Analyzer 1110, Thermo Scientific GmbH) after pre-treatment with a 1:1 HCl solution to eliminate traces of carbonates. Microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) were determined within a week of soil collection on field moist soil samples stored at +4 °C using the chloroform-fumigation extraction method (Brookes et al., 1985). Organic carbon and total nitrogen content were determined with an elemental analyzer (TOC-VCPH/CPN, Shimadzu). The organic carbon content of the unfumigated extracts was used as an estimation of soil extractable organic carbon (C_{extr}).

The activity of eight extracellular hydrolytic enzymes was determined using MUF conjugates at final concentrations ensuring substrate saturating conditions (Giacometti et al., 2013, submitted) (Table 1). The assays were conducted on deeply frozen soil samples (−20 °C) within 4 months after soil sampling (ISO/TS 22939, 2010). A 0.5 M sodium acetate buffer solution at pH 5.5 was used to dilute standard, substrates and soil samples. Standard and substrate solutions were prepared on the day of the assay and kept in the dark until use. To avoid microbial contamination, glassware, buffer, and deionised water were autoclaved before use (121 ± 3 °C for 20 min.). Substrates were pre-dissolved in DMSO (dimethyl sulfoxide, Sigma). Sodium acetate buffer was then added to reach the desired final concentration (Table 1). Five mM 4-methylumbelliferone (MUF) standard solution was prepared in methanol and deionised water (1:1, v:v) and diluted to 1.00, 2.00, 4.00, 10.0, 20.0, 30.0, 40.0 μM in sodium acetate buffer. The equivalent of 4 g of oven dried soil was suspended in 100 mL of 0.5 M acetate buffer and mixed with an Ultra Turrax (IKA-Werke, Staufen, DE) for 2 min at 9000 rpm. Soil assay (100 μL soil slurry + 100 μL substrate solution), substrate controls (100 μL substrate solution + 100 μL buffer), reference standards (50 μL standard solution + 150 μL buffer), quench controls (100 μL soil slurry + 50 μL standard solution + 50 μL buffer) and soil controls (100 μL soil slurry + 100 μL buffer) wells were set up in flat-well black polystyrene 96-well microplates following the order reported by DeForest (2009). Microplates were covered and incubated in the dark at 30 °C. Fluorescence intensity was measured using a microplate fluorometer (Infinite® 200, TECAN, Männedorf, CH) with 365 nm excitation and 450 nm emission filters. Measurements were taken immediately after the plate set-up and then every 30 min over a 3 h incubation period. Before each reading the microplates were shaken for 5 s to homogenize the reaction mixture. Rates of fluorescence increase were converted into enzyme activity (nmol MUF g^{-1} h^{-1}) according to German et al. (2011).

Results were expressed on an oven-dried basis. All statistical analyses were carried out in the R environment (R Core Team, 2013). The experimental data was analyzed as a split-plot arrangement of treatments with organic fertilization as main factor and mineral N fertilization as split factor Organic fertilization marginal means were calculated on the average of N fertilization rates. Similarly, N fertilization marginal means were calculated on the average of organic fertilization treatments. Means were separated by calculating Bonferroni adjusted least significant difference (LSD). Relationships among soil properties were estimated using simple linear regression. Pearson's product moment correlation coefficients (r) and significance of the associations (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$) are reported. Principal component analysis (PCA) was carried out to reveal differences among treatments. Pearson's product moment correlation coefficient was used to evaluate the correlation between the first two principal components and the original variables.

Data on soil chemical and microbiological properties are reported in Table 2. Soil pH was not significantly affected by organic or mineral N fertilization. However, differences were observed between blocks: soil samples from Blocks 1 and 4 had relatively neutral pH values (pH 7.4 and 6.5, respectively), while soil samples from Blocks 2 and 3 were more acidic (pH 5.6 and 5.7, respectively). Small amounts of carbonate (<4.00 g kg^{-1} $CaCO_3$) were detected in some samples and were probably responsible for the differences in soil pH observed between blocks. In a previous study using samples from the same long-term experiment (Giacometti et al., 2013), mineral N fertilization reduced soil pH (Clegg, 2006). However, the effect was evident only shortly after N fertilization. In the present study soil samples were collected in March, before maize sowing and urea application, and no clear effect of N fertilization on soil pH was observed.

In accordance with Rasmussen and Collins (1991) and Reeves (1997) manure's positive effects on SOM exceeded that of crop residue: long-term fertilization with manure increased SOC and TN while no significant differences were observed between the control and the crop residue treatments. With regard to the organic carbon extractable fraction (C_{extr}) values were minimum in the plots amended with crop residue and maximum in the plots amended with manure. Conversely, the highest C:N ratio was observed under crop residue amendment. These results are in accordance with previous observations and suggest that the quality of the organic inputs added influenced the quantity of SOM accumulating under the different treatments as well as its chemical composition and availability (Marschner et al., 2003; Giacometti et al., 2013).

Treatments amended with manure showed higher values of C_{mic} as compared to the crop residue and control treatments. Similar results were observed for N_{mic}, even though in this instance crop residue showed intermediate values between control and manure treatments. The small changes in the C_{mic}:N_{mic} ratio observed under the different treatments may indicate a shift in microbial community composition due to organic and mineral N fertilization (Zelles, 1999; Giacometti et al., 2013). C_{mic} and N_{mic} were positively correlated with SOC, TN and C_{extr} (Table 3), indicating that microbial proliferation was strongly related to the quantity of substrates available for growth. Significant effects of the subplot factor mineral N fertilization were not observed (Table 2 and S1).

Data on soil enzyme activities are reported in Fig. 1. β-Cellobiosidase appeared affected by organic fertilization, with higher activity in the manure treatment compared to crop residue and control treatments (+30%). Similarly, β-glucosidase activity was higher under manure treatment as compared to crop residue and control treatments (+35% and +60%, respectively). Similar results were observed in samples analyzed following the colorimetric procedure of Eivazi and Tabatabai (1988) (Giacometti et al., 2013). A trend similar to β-cellobiosidase and β-glucosidase was observed for β-xylosidase, even though differences were not statistically significant (Table S1). Other investigations (Kandeler et al., 1999; Böhme et al., 2005; Marinari et al., 2006; Moscatelli et al., 2012) have also reported positive effects of manure amendment on soil β-glycosidases. In accordance with Dodor and Tabatabai (2005) the activities of these glycosidases were positively correlated with total and extractable organic C as well as with TN and microbial biomass C and N (Table 3). These correlations confirm that soil enzymes are mainly a function of the amount of substrate available (Kandeler et al., 1999) and of the microbial biomass present to potentially synthesize them (Geisseler and Horwath, 2009).