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Enzyme Measurements Increases Understanding of Effects of Land Management Practices

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ABSTRACT

A Hanslope Soil health was investigated from context of enzyme activities namely: cellulose, phosphomonoesterase and urease to enhance the understanding of the following land management practices: grassland under permanent pasture on 5 y ley to stocking, another sown with red clover a y before stocking, the other fertilized with N-fertilizer a y to stocking; the last on barley; with pristine deciduous woodland as control. The highest cellulose activity was detected in deciduous woodland, 172.3 ± 106 µg glucose g^{-1} d^{-1} over the summer. Cellulase activity was positively dependent on soil microbial C (R^2 = 0.64). Grassland under permanent pasture treated with N-fertilizer a y before stocking had the highest urease activity, 77 ± 57.8 µg NH_4-N g^{-1} h^{-1} over winter in both buffered and non buffered measurement techniques. There was no significant differences in phosphomonoesterase activity between land management. Soil enzyme measurements is a major component of soil health. It defines biological productivity, promote environmental quality and maintains plant and animal health. Soil health is fundamental for agricultural sustainability. Amongst the soil biological properties, enzymes show extraordinary specificity in catalyzing biological reactions as revealed in this study. Regional information are solicited for global network.

Introduction

Enzyme measurements answer qualitative questions about specific metabolic processes, and in combination with other measurements (ATP,CO_2 evolution) increases the understanding of the effect of agrochemicals, cultivation practices, environmental and climatic factors on the microbiological activity of soil (Skujins, 1978; Nannipieri, 1994; Alef and Nannipieri, 1995). Curci *et al* (1977) cited by Girvan *et al* (2003 discovered high enzyme activity in the uppermost 20 cm of soil in plots tilled by shallow ploughing.

Enzymes show extraordinary specificity in catalyzing biological reactions. Research into soil enzymes has increased over the past 30 years. Various activities associated with biotic and abiotic components contribute to the overall activity of soil enzymes. According to Burns (1982) an enzyme may be associated physically with proliferating animal, microbial and plant cells and it may be located in the cytoplasm in the periplasm of gram negative bacteria or attached to the outer surface of cells. They can also be present in non-proliferating cells, for example, microbial spores or protozoan cysts, in entirely dead cells or in cell debris.

Other enzymes are present as an extracellular soluble molecule. They can also be temporarily associated in enzyme-substrate complexes, adsorbed to clay minerals or associated with humic colloids. Some of these categories according to Alef and Nannipieri (1998) may represent various stages in the life of an enzyme. An intracellular enzyme may still function after the cell dies and thus it becomes associated with cell debris. It may be released in the aqueous phase and may eventually be adsorbed in an active form by soil colloids

Enzyme-clay and enzyme-organic polymer complexes show remarkable resistance to proteolytic and thermal denaturation (Sarkar *et al*, 1980; Burns, 1982, 1986; Trasar-Cepeda and Gil-Sotres, 1987, 1988; Nannipieri *et al*, 1988). Several methods exist for the measurement of enzyme activities. These measurements represent the maximum potential because the incubation conditions for enzyme assays are chosen to ensure optimal rates of catalysis. Thus, the concentration of substrate is in excess and the optimum values of pH and temperature are selected to permit the highest rate of enzyme activity. Also the volume of the reaction mix is such that it allows free diffusion of substrate. Hence, the problems arising from the interpretation of measured soil enzyme activity have often led to the conclusion that soil enzyme assays have no meaning in ecological and agricultural terms (Nannipieri, 1994).

Nevertheless, it has. It helps define biological productivity, promotes environmental quality and maintain plant and animal health that are vital for agricultural sustainability. This is the basis of soil health studies. The challenges are numerous. Soils suffer from a mix of physical degradation by water and wind erosion, crusting, sealing and waterlogging, chemical degradation by acidification, nutrient depletion, pollution from industrial wastes and excessive use of pesticides and fertilizers, loss of soil flora and fauna (Pretty, 2002). Soil health can be improved through a variety of practices including the use of legumes, green maures and cover crops, the incorporation of plants with the capacity to release P from the soil and the use of composts and animal manures. In addition to the adoption of zero-tillage, inorganic fertilizers may be used as a sustainable agricultural practice (Pretty, 2002; Briggs, 1989; Reijintjies, 1997; Vlaming et al, 1997; Bunch, 1997; Benzing, 1997; Mullerriyawa and Wettasinha, 1997; Zakaria and Laban, 1997; Wanjau et al, 1997; Sharland, 1997).

Enzyme activity measurements increases understanding of the effects of land management practices. Evidence from scientific literature shows the degradation of cellulose in soils is

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a slow process which depends on concentration, location and mobility of cellulases. Other factors include type of litter, substrate concentration, pH, temperature and water (Hunt, 1977; Sinsabaugh and Linkins, 1988; Tateno, 1988; Alef and Nannipieri, 1998; Kshattriya *et al*, 1992). Many factors influence urease activity: resistance to thermal and proteolytic denaturation(Burns *et a*l, 1972; Nannipieri *et al*; 1974); enzyme-organo-mineral complexes (Nannipieri *et al*, 1978a), soil drying, irradiation and storage temperature (McLaren, 1969; Pancholy and Rice, 1973; Zantus and Bremner, 1975b; Kandeler and Gerber, 1988; Fenn *et al*, 1992. Others include soil pH and the type of buffer used in the assay (Tabatabai, 1977; Nor, 1982; Tabatabai, 1982; Doelman and Haanstra, 1986; Kandeler and Gerber, 1988; Cochran *et al*, 1989; McCarty and Bremner, 1991; McCarthy *et al*, 1992). Nannipieri *et al* (1978a) noted resistance of enzyme-organo-mineral complexes with high molecular weights compared to those with lower molecular weights.

Generally, the activity of phosphomoesterase is strongly influenced not only by pH and temperature but also by the OM content, soil moisture and anaerobiosis (Alef et al, 1988). The current study is an addition to this subject matter as scientists profer alternative solutions to assessing soil health and agricultural productivity.

Materials and Methods

Site Description

The project area is Writtle College, UK research and teaching farm located within 51° 44¹, 0° 26¹ E, 32 OD, 68 klm east of London. The soil belongs to Hanslope Soil Series of Chalky Boulder Clay parent material. It is a grassland under permanent pasture: one on 5 y ley before stocking, the other sown with red clover a y to stocking, the third treated with N fertilizer a y before stocking, the fourth under barley and deciduous woodland as control. Each site had four replicate sampling points which were revisited on each sampling time.

Laboratory protocols

Cellulase activity

Cellulase activity was estimated using the method described by Deng and Tabatabi (1994). Air dry soil (5 g) was placed in a centrifuge tube (25 ml) followed by the addition of toluene (250 µl) buffered CMC (carboxylmethyl cellulose) (20 ml) and incubated at 30°C for 24 h. After incubation the soil suspension was centrifuged 3 times at 17,390 g (1000 rpm) for 10 min. The supernatant (500 µl) was placed in a test tube followed by the addition of DNS (dinitrosalicyclic acid, 500 µl). Tubes were boiled in a water bath at 100°C for 10 min. Once cooled deionised water (2.5 ml) was added. The absorbance was read at 540 nm in a spectrophotometer. Glucose standards were prepared by adding glucose (500 µl) obtained by dissolving 28 mg of glucose monohydrate in deionised water (1000 ml) to DNS reagent (500 µl). Tubes were boiled for 10 min at 100°C and allowed to cool before the addition of deionised water (2.5 ml). The absorbance was read at 540 nm in a spectrophotometer. Similarly, a blank solution, was prepared by adding deionised water (500 µl) to DNS reagent (500 µl), boiling for 10 min and adding deionised water (2.5 ml). The absorbance at 540 nm was read in a spectrophotometer. For the calibration curve 0, 0.05, 0.1, 0.15 and 0.2 mg l^{-1} of monohydrate glucose was prepared. Calculation of cellulose activity (µg g^{-1} dwt 24 h^{-1}) = C x v x f/Sw x dwt Where C is glucose concentration (absorbance reading of soil sample)

v is volume of suspension (10 ml)

f is dilution factor

sw is the amount of soil taken (5 g)

dwt is the dry weight of 1 g soil sample

Phosphomonoesterase activity

The method for the estimation of phosphomonoesterase activity was based on that described by Tabatabai and Bremner (1969) and Livavi and Tabatabai (1977). For the assay of acid phosphate air dry soil (1 g) was placed in an erlenmeyer flask (50 ml) and treated with toluene (0.25 ml), MUB (modified universal buffer, pH 6.5 (4 ml) and p-nitrophenyl phosphate (PNP) solution made in the same buffer (1 ml). For the assay of alkaline phosphatase the same volume of soil and reagent was used except that MUB (pH 13) was used with PNP solution made in the same buffer (1 ml). After stoppering the flasks the contents were mixed and incubated for 1 h at 37°C $CaCl_2$ (0.5 M, 1 ml) was added together with 0.5 M NaOH (4 ml). The contents were mixed and soil suspension filtered through Whatman No 2 filter paper. The absorbance of the filtrate was measured at 400 nm in a spectrophotometer. For the controls the PNP solution was added was added after the addition of 0.5 M $CaCl_2$ and 0.5 M NaOH (4 ml) and immediately before the filtration of the soil suspension. All measurements was performed in triplicate. For the calibration curve standard p-nitrophenol solution (1 ml) was diluted with deionised water (100 ml) in a volumetric flask. Six separate aliquots of the diluted standard solution (0, 1, 2, 3, 4 and 5 ml) were pipette into Erlenmeyer flask (50 ml) before the adjustment of the volume with deionised water (5 ml). Finally, the same process as described for PNP analysis of the incubated soil samples was followed. The results were corrected for the control before the p-nitrophenol content (ml^{-1}) of the filtrate was calculated by reference to the calibration curve and the following equation

p-nitrophenol (µg g^{-1} dwt h^{-1}) = C x v/dwt x sw x t

where C is the measured concentration of p-nitrophenol (µg ml^{-1} filtration)

dwt is the dry weight of 1 g moist soil

v is the total volume of the soil suspension in ml

sw is the weight of soil sample used (1 g)

t is the incubation time in h

Urease activity

Urease activity in the soil was estimated using the method decribed by Kandler and Gerber (1988). For the non-buffered method moist soil (5 g) was measured into Erlenmeyer flasks (100 ml) followed by the addition of 200 mM urea solution (2.5 ml). The flask was stoppered and incubated at 37°C for 2 h. After incubation 2.5 M KCl solution (50 ml) was added followed by shaking the flask for 30 min, with a mechanical shaker. This was followed by the filtration of the resulting suspension. The ammonium content was nalysed as follows: the clear filtrate (1 ml) was pipette into an Erlenmeyer flask (50 ml) followed by the addition of deionised water (9 ml) and 0.3 M Na-salicylate/NaOH solution (5 ml) and 0.1 M Na-dichlorocyanurate solution (2 ml concentration). The soil suspension was allowed to stand at room temperature for 30 min. The optical density of solution was measured at 690 nm. The reading was taken immediately after the addition of Na-dichloro cyanurate solution in the soil extract. The blanks were prepared by adding deionised water (2.5 ml) to an erlenmeyer flask (100 ml); the flask was stoppered and incubated at 37°C for 2 h. After incubation 200 mM urea solution (2.5 ml) was added together wit 2.5 M KCl solution (50 ml). The flask was shaken for 30 min followed by the analysis of ammonium content as follows: the filtrate (1 ml) was pipette into an Erlenmeyer flask (50 ml) followed by the addition of deionised water (9 ml), 0.3 M Na-salicylate solution (5 ml) and 0.1 M Na-dichloro cyanurate solution (2 ml). The mixture was allowed to stand for 30 min at room temperature. The optical density was immediately

measured at 600 nm using a spectrophotometer The procedure for the buffered method was as follows: Moist soil (5 g) was placed in an Erlenmeyer flask followed by addition of 200 mM urea solution (2.5 ml) and 0.3 M borate buffer (200 ml). The flask was stoppered and incubated at 37°C for 2 h. After incubation 30 ml of 2.5 M KCl was added before shaking for 30 min. The resulting suspension was filtered and analysed for ammonium as follows: the clear filtrate (1 ml) was pipette into an Erlenmeyer flask (50 ml) followed by the addition of deionised water (9 ml) and 0.3 M Na-salicylate/NaOH solution (50 ml) and 0.1 M Na-dichlorocyanurate solution (2 ml). The mixture was allowed to stand for 30 min at room temperature. The optical density was read in 690 nm For the preparation of the calibration curve, ammonium standard (0, 1, 1.5, 2, 2.5 µg NH_4-N ml^{-1} was pipette into test tubes followed by dilution with deionised water (9 ml). The NH^4 concentration was determined at 690 nm following the protocol described for the soil sample filtrate Urease activity (µg NH^4-N g^{-1} dwt 2 h^{-1}) was determined as follows

µg NH_4-N ml^{-1} x v x 10/dwt x 5

where dwt is the dry weight of 1 g moist soil

v is the volume of extract (52.5 ml) which can vary

dilution factor is 10

weight of soil sample used is 5 g

Statistical and Data Analysis

The differences in cellulose, urease and phosphomonoesterase between land management activities were based on two way ANOVA and Fishers Least Significant Difference (FLSD). Effects at P≤0.05 was considered significant while at P≤0.01 was considered highly significant (Zar, 1999). Cellulase activity was regressed with soil microbial C (SXSTAT11.EXE version 1.0.7). Goodness of fit from predictions with each of the regression equations against actual values were expressed with the coefficient of determination (R^2).

Results and Discussions

Cellulase activity

There was high significant differences (F = 476; FLSD = 19, P≤0.01) in cellulose activity between land management practices over winter (Dec – Feb). The highest cellulose activity across months was in soil collected from deciduous woodland, 172.3 ± 106 µg glucose g^{-1} d^{-1} followed by soil collected from grassland under permanent pasture treated with N fertilizer a y before stocking, 50.2 ± 60.3 µg glucose g^{-1} d^{-1}; the least cellulose activity was in arable land under barley, 26.1 ± 35.1 µg glucose g^{-1} d^{-1}. Overall, soils sampled (Jun – Aug) in summer showed highest cellulose activity, 101.4 ± 62.1 µg glucose g^{-1} d^{-1}; while the lowest activity was detected in samples collected at spring (Mar – May), 23.1 ± 13.3 µg glucose g^{-1} d^{-1}. Cellulase activity was positively correlated to soil microbial C (R^2 = 0.7, n=12).

Urease activity

There were significant differences in urease activity between land management (F = 20.2, FLSD = 2.99, P≤0.01) over spring in the non-buffered method and autumn in the buffered method (F = 38.2, FLSD = 138.9, P≤0.01). Soil from the grassland under permanent pasture treated with N fertilizer a y before stocking gave the highest urease activity across months under both measurement techniques, 77 ± 57 and 181.4 ± 133.5 µg NH4-N g^{-1} h^{-1}. The highest urease activity was recorded during winter in the non-buffered method, 119.4 ± 17.3 µg NH4-N g^{-1} h^{-1}, while the lowest activity recorded under the same measurement technique over spring, 10.7 ± 2.1 µg NH4-N g^{-1} h^{-1}. In the buffered measurement the highest urease activity was recorded over autumn, 211.3 ± 131.9 NH4-N g^{-1} h^{-1} while the lowes activity was in spring, 16.4 ± 1.1 NH4-N g^{-1} h^{-1}.

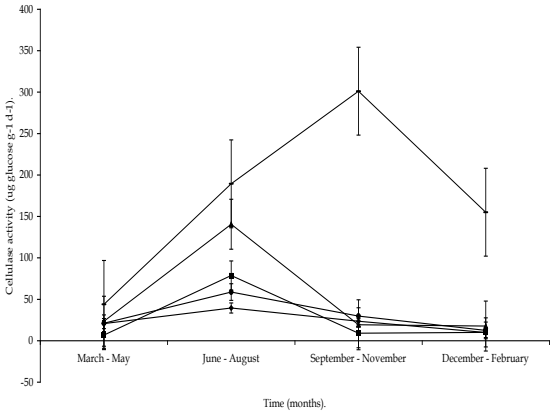

Fig 1. Monthly time course cellulose activity (µg glucose g^{-1} d^{-1} in soil under:♦ grassland under permanent pasture on 5 y ley before stocking; ●grassland under permanent pasture sown with red clover a y before stocking; ▲grassland under permanent pasture treated with N fertilizer a y before stocking; ■arable land under barley; ═deciduous woodland. Each value is mean of 4 relicates x 3 months. Bars represent standard error of means

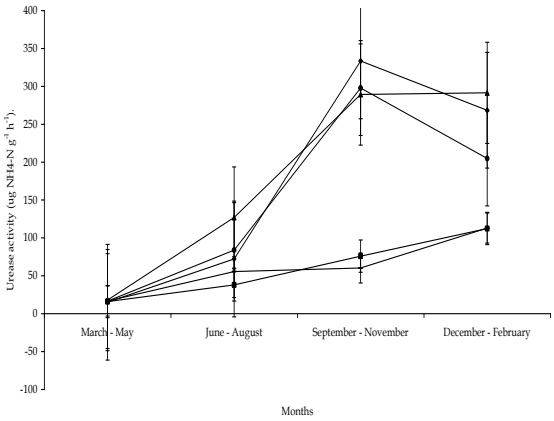

Fig 2. Non-buffered urease activity measurement under different management practices as detailed in Fig. 1.

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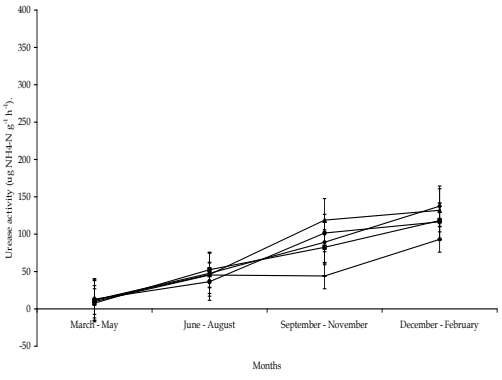

Fig 3. Buffered urease activity under different management practices as detailed in Fig 1

Phosphomonoesterase activity

There was no significant differences in phosphomonoesterase activity despite land management practices and temporal variations. However, the highest activity was found in soils from the deciduous woodland, 27.4 ± 6.8 µg g^{-1} h^{-1} p-nitrophenol. The lowest levels of activity was detected in soils from the grassland under permanent pasture treated with N fertilizer a y before stocking, 23.2 ± 4.5 µg g^{-1} h^{-1}.The highest activity was recorded over winter, 30 ± 4.1 µg g^{-1} h^{-1} p-

nitrophenol, while the lowest activity was in spring, 19.6 ± 1.3 µg g^{-1} h^{-1} p-nitrophenol.

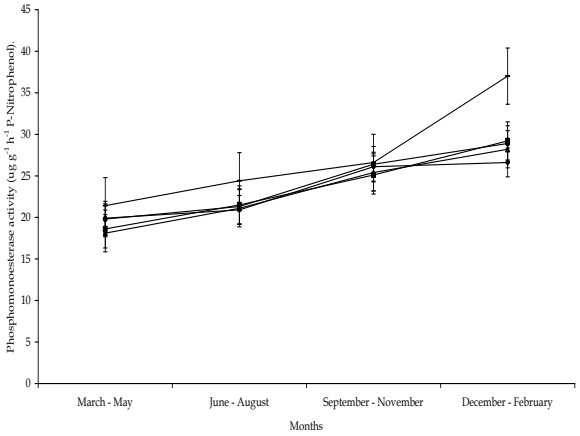

Fig 4. Monthly time course phosphomonoesterase activity under various land management activities as detailed in Fig 1

The high cellulose activity in soils from the deciduous woodland, the summer months and significant correlation between cellulose activity and soil microbial carbon may be due to several factors. Evidence from literature show that degradation of cellulose in soils is a slow process which depends on concentration, location and mobility of cellulases. Other factors include type of litter, substrate concentration, pH, temperature and water concentration (Hunt, 1977; Sinsabaugh and Linkins, 1988, Tateno, 1988; Alef and Nannipieri, 1998; Kshattriya *et al*, 1992) Benefield, 1971; Pancholy and Rice, 1973; Hope and Burns, 1987; Alef and Nannipieri, 1998 reported cellulose activity of soil as optimum at pH 5 – 6 and a temperature ranging from 30 to 50°C. These workers observed an increase in cellulose activity in rhizosphere soil as compared to the activity of non-rhizosphere soil. Similarly Curci et al (1997) and Girvan *et al* (2003) reported enzyme activity was higher in the uppermost 20 cm of soil in plots tilled by shallow ploughing and scarification. Temperature appear to be the dominant factor controlling cellulose activity in summer while soil pH may have contributed to the cellulose activity in grassland under permanent pasture treated with N fertilizer a y y to stocking. On the other hand the type of litter, substrate and concentrations may be the deciding factors in the deciduous woodland. It's litter type (probably in cellulose content, even though not determined), the substrate concentration that was high based on over 100 years of litter accumulation as pristine woodland, and the capacity to retain water based on organic matter. However, it is likely that the cellulose activity quoted represent values near to the maximum rates and not the actual rates of cellulose activity in the soil

The highest urease activity in grassland pasture treated with N fertilizer a y before stocking may be due to presence of nutrients (particularly ammonium) while the low urease activity in deciduous woodland and in arable land under barley may be due to several factors. I t may be higher in soil samples collected over the winter months due to a reduction in ammonia loss via volatilization, while it may be lower in soil samples collected over the spring months due to lower ammonium concentration in the soil as a result of increased temperature and ammonia volatilisation. Other factors that influence urease activity are resistance to thermal and proteolytic denaturation (Burns, *et al*, 1972; Nannipieri *et al*, 1974); enzyme organo-mineral complexes (Nannipieri et al, 1978a), soil air drying, irradiation and storage temperature (MacLaren, 1969; Pancholy and Rice, 1973; Zantua and Bremner, 1975b; Kandeler and Gerber, 1988; Nor, 1982; Tabatabi, 1982; Doelman and Haanstra, 1986; Kandeler and Gerber, 1988; Cochran et al, 1989, McCarty and Bremner, 1991; McCarty, et al, 1992). For example, Burns [et al] (1972) and Nannipieri *et al* (1974) observed ureases extracted from soils to be resistant to thermal and proteolytic denaturation with Nannipieri *et al* (1978a) relating resistance of enzyme-organo-mineral complexes with high molecular weights compared to those with lower molecular weights. From results the urease activity was not limited by thermal denaturation. A temperature optimum as high as 60°C has been reported, urease being denatured at 70°C while assays have generally been carried out in the temperature range 15 - 42°C. However, soils are incubated at 30°C (Summer, 1951; Zantua and Bremner, 1977; Bremner and Mulvaney, 1978; Kissel and Cabrera, 1988; Moyo *et al*, 1989). This incubation condition was also taken into consideration in this work. With regards to the optimum pH range for urease activity published results vary: both neutral (pH 6 – 7) – Hoffman and Schmidt, 1953; and alkaline pH (8.8 – 10) have been reported as optimum (Tabatabi and Bremner, 1972; May and Douglas, 1976; Kandeler and Gerber, 1988; Perez-Mateos and Gonzalez-Carcedo, 1988). In this work soil from the grassland under permanent pasture treated with N fertilizer a y before stocking showe neutral pH, that from arable land under barley was alkaline, while that from deciduous woodland was acidic. In terms of relative molecular weight of soil organic matter and their role on urease activity may have been limited in deciduous woodland in view of likely molecular weight of the fractions compared to those samples from the N plot. Hence, the higher urease activity recorded in this type of land management practice over the period. However deciduous woodland has other great potential. For example, Burns et al (1972) saw a tendency of urease-organic-complexes of high molecular weight to possess arrangements that permit the movement of substrates and products towards the enzyme but not that of large molecules such as proteases. A comparison of urease activity assessed using either a buffered or non-buffered systems showed that buffered results was higher than non-buffered to the tune of 100%. Similar results were reported by Kandeler and Gerber, 1988. According to Tabatabi (1982) the Tris buffer used in the assay prevents NH_4^+ fixation by soil

Phosphomonoesterase activity did not vary either temporarily or between management practices. There is however criticism over the use of pheoltic esters as substrates as they might not be suitable for assaying the hydrolysis of natural esters such as inositol phospahates and nucleotides (Alef and Nannipieri, 1988). Tabatabi and Bremner (1969), Eivazi and Tabatabai (1977) cautioned on the determination of phosphomonoesterase activity under these conditions in acid soils as the p-nitrophenyl phosphate is rapidly hydrolysed to p-nitrophenol under acidic conditions. It s recommended that enzymatic reaction be stopped by placing soil suspension on ice at the end of the incubation (Nannipieri et al, 1978a,b; Gadkari, 1984). Likewise, the addition of $CaCl_2$ to prevent dispersion of clays during the subsequent treatment with NaOH (Alef and Nannipieri, 1998). Caution is also required in choice of buffers as soil phospahate activity is inhibited by acetate and citrate-phosphate buffers 9Speir and Ross, 1978). The inhibition by citrate-phospahate buffer is related to the effect of phosphate (Malcolm, 1983). Inorganic extraction of p-nitophenol has also been observed in soils with high OM content and extractable Fe and Al. A quantitative recovery of phenol released from phenylphosphate was observed in these soils (Harrison, 1979). Generally, the activity of phosphomonoesterase is strongly

influenced not only by pH and temperature values but also by the OM content, soil moisture and anaerobiosis (Alef et al, 1988). Hence, due to these effects phosphomonoesterase activity has been shown to vary seasonally (Speir and Ross, 1978; Beck, 1984a; Sparling et al, 1986; Pulford and Tabatabai, 1988; Rastin et al, 1988. However, these variations were not detected in significant levels in this study

Conclusion

It has been established that enzyme activities help explain effects of land management practices. As cellulose acitivity defined deciduous woodland; urease activity defined effects of grassland under permanent pasture treated with N fertilizer a y before stocking and phosphomonoesterase activity could not explain effects of any of the land management practices. Nevertheless, it remains a popular choice for estimating soil health and overall agricultural capacity and productivity of agricultural soils

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