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ORIGINAL PAPER

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Response of ATP content, respiration rate and enzyme activities in an arable and a forest soil to nutrient additions

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Abstract Glucose (C), glucose plus NO_3^- (C+N) or glucose plus NO_3^- plus PO_4^{3-} (C+N+P) were added to an arable and a forest soil at a single dose, or split into four equal doses over 4 consecutive days, and the response of several enzyme activities, ATP content and respiration rate were monitored for 11 days. ß-Glucosidase activity was reduced in the two soils during the first day by substrate addition. Thereafter, this enzyme activity varied only slightly in the arable soil with reference to the non-amended control, while it increased substantially in the beech forest when C+N and C+N+P were added. Casein-hydrolysing activity increased in the C treatment and decreased after C+N+P addition during the first 4 days in the two soils. After 11 days, protease activity was enhanced in the arable soil when C+N was applied in a split dose. Urease activity decreased during the first 4 h, particularly in the arable soil with the addition of C+N or C+N+P, applied in a single dose, and then continuously increased. Thus, urease responded to high nutrient availability, being firstly repressed or inhibited, and stimulated afterwards. Phosphatase activity was only slightly modified in the arable soil but substantially increased in the beech forest by C+N addition. The presence of P usually decreased phosphatase activity. Arylsulphatase activity was repressed after substrate addition, which was particularly evident in the arable soil. In the beech forest topsoil, C added alone increased this enzyme activity. Significant correlations between ATP content and enzyme activity were only observed for urease in the arable system treated with C+N and in the forest when C was applied in a split dose. The effect of C, C+N and C+N+P addition varied between the arable and the forest soil according to environmental conditions and microbial ecophysiology.

Keywords Arable · Enzyme · Extracellular · Forest · Induction

Introduction

The activity of any enzyme in soil is not always controlled by the respective protein in active cells. Many soil enzymes, particularly those hydrolysing organic compounds, are produced by plants, animals and, in many cases, predominantly by microorganisms, and may be present in dead cells and cell debris and also adsorbed by clay or incorporated into humic substances (Burns 1978, 1982; Nannipieri et al. 1990, 1996a; Skujins 1978). Therefore, enzymes can be present in the absence of proliferating and non-proliferating microorganisms. Extracellular enzymes can be stabilised over long periods by their interactions with the soil matrix and may, therefore, be insensitive to environmental conditions that affect microbes. Thus, enzyme activities can be regarded as an enzymatic memory of the catalytic history of a soil. In contrast, microorganisms are suggested to respond sensitively to changing environmental conditions and adjust their enzymatic repertoire accordingly. The most serious problem for the interpretation of measurements of enzyme activities is deciding which combinations of enzyme activities have been experimentally determined (Burns 1982; Nannipieri et al. 1996b). The distinction between intracellular and extracellular activities is, therefore, important, and may allow the separation of the relative contribution of the stabilised and resistant fraction from that of sensitive intracellular enzyme fraction (Dilly and Nannipieri 1998; Klose and Tabatabai 1999; Nannipieri et al. 1996b, 2000).

Microbial activity in soil is affected by the availability of easily decomposable organic substrates (Friedel et al. 1996; Klose et al. 1999). Glucose is one of the substrates readily degradable in soil, and its addition to soil

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generally increases microbial activity and stimulates microbial growth within a few hours, as indicated by the increase in soil respiration, enzyme activities and microbial biomass in glucose-treated soils (Anderson and Domsch 1973; Drobnik 1960; Lin and Brookes 2000; Nannipieri et al. 1978, 1979; Sparling 1995). Micro-calorimetric measurements showed that glucose uptake rates in a humic Cambisol increased up to a concentration of 15 mg g^{-1} soil per day (Barja and Nunez 1999). Further increases in glucose addition caused a decrease in the microbial growth rate, which was hypothesised to be due to an inhibitory effect on the activity of specific microbial permeases by excess glucose. Nutrient shortage has been demonstrated to be inhibitory of soil respiration rates at high glucose concentrations (Stotzky 1965).

Nannipieri et al. (1996b) used the McLaren and Pukite (1973) approach to determine the extracellular phosphatase activity at pH 6.5 in three soils which differed in the amount of sewage sludge applied annually for 8 years. The soil enzyme activity increased by adding glucose and NO_3^-, and it was significantly correlated to the ATP content. At zero ATP a positive intercept of phosphatase activity was observed, which was assumed to be that of the extracellular component. This method for determining the extracellular activity of an enzyme in soil has been termed the "physiological method" (Nannipieri et al. 2000). However, drawbacks such as the non-coincidence of the increase in the enzyme activity with microbial growth and the partial response of soil microbiota to nutrient addition have been assumed (Dilly and Nannipieri 1998; Nannipieri et al. 2000). Indeed, the synthesis of enzymes may be repressed or induced by the presence of particular compounds without a change in microbial biomass. Only the activity of constitutive enzymes, which are not subjected to this regulation, can be related to microbial growth. It has been shown that different enzymes do not respond synchronously when the soil is treated with C and N sources; peaks of phosphatase and urease activity preceded an increase in caseinhydrolysing activities regardless of the kind of C source added to the soil to stimulate microbial growth (Nannipieri et al. 1983). In view of the very small amount of work done, further research is needed to verify the validity of the approach proposed by Nannipieri et al. (1996b) to distinguish the extracellular from the intracellular enzyme activity in soil. Therefore, this approach has to be tested with different soils and enzymes other than phosphatase (Nannipieri et al 2000).

The aims of this work were: (1) to study the temporal response of the activities of several hydrolases involved in C, N, P and S cycling, such as β-glucosidase, urease, phosphatase, arylsulphatase and casein-hydrolysing enzymes to the addition of growth-limiting C, N and P substrates; (2) to test the correlation of these activities to the ATP content as an indicator of microbial biomass; and (3) to find the optimum conditions for setting up a method to determine the extracellular activity of these enzymes in soil.

Materials and methods

Site and soils

Two contrasting soils from the long-term ecosystem research programme were used to evaluate the response of contrasting microbial communities to nutrient addition (Dilly and Munch 1998). They were located at the experimental site of the interdisciplinary project Ecosystem Research in the Bornhöved Lake District (54°06′N, 10°14′E). The landscape, formed during the Pleistocene, consists of morainic hills and lakes. The climate was influenced by the North Sea and the Baltic Sea. The long-term (1951–1980) mean annual rainfall was 697 mm and the average annual air temperature was 8.1°C.

The agricultural soil was under crop rotation (mixture of *Lolium perenne* L. and *Festuca rubra* L. in the sampling year), fertilised with organic manure previously and with 40 kg KNO_3-N ha^{-1} in the sampling year. The forest soil was under beech (*Fagus sylvatica* L.). The forest topsoil was located below a litter horizon of approximately 5 cm thickness. The arable soil type was an Eutri-cambic Arenosol (FAO 1988) with a pH of 6.4, an organic C (C_{org}) of 14.4 mg C g^{-1} dry soil, a C/N ratio of 10 and a basal respiration of 26 µg CO_2-C g^{-1} C_{org} h^{-1} estimated during 11 days at 22°C. The beech forest soil type was a Dystri-cambic Arenosol with a pH of 4.1, an C_{org} of 33.8 mg C g^{-1} dry soil, a C/N ratio of 14 and a basal respiration of 19 µg CO_2-C g^{-1} C_{org} h^{-1}.

From three different locations at each site, soil was sampled from the A horizon at approximately 20 cm depth for the arable and 5 cm for the forest soil. Multiple cores (20 cores at the arable field taken with a 2-cm-diameter Puerckhauer drill, 10 cores at the beech forest taken with a 4-cm-diameter drill to minimise the translocation of material from the litter horizon to the topsoil during sampling) were sampled, mixed together separately for each location and site, visible plant materials were removed, the soil sieved at 2 mm and stored at 4°C until analysis. The experiment was started within 2 weeks after sampling. Since soil water content varied between 40% and 70% of the water holding capacity at sampling, moisture did not limit respiration rates in situ, and drying or rewetting of the soils was not necessary.

Experimental design and analyses

Fresh soil (100 g) was weighed at the bottom of 1-L jars containing two vessels with 5 ml deionised water for air humidifying and sufficient 1 M NaOH for CO_2 adsorption and the determination of the respiration rates. Soil was pre-incubated for 3 days at 22°C to mineralise readily degradable organic compounds and to stabilise microbial activity. Then, substrates containing C, N and P were added (three replicates each). Six treatments were considered: soils were treated with only glucose, equivalent to 2 mg C g^{-1} dry weight (wt) soil, added in a single dose [C(1)], or split into four equal dose at 0, 24, 48 and 72 h [C(4)]; soils received glucose and KNO_3 equivalent to 2 mg C and 0.25 mg N g^{-1} dry wt soil, respectively, added in a single dose [C+N(1)], or split into four equal doses applied at 0, 24, 48 and 72 h [C+N(4)]; soils received glucose, KNO_3 and KH_2PO_4 equivalent to 2 mg C, 0.25 mg N and 0.125 mg P g^{-1} dry wt soil, respectively, added in a single dose [C+N+P(1)], or split into four equal doses applied at 0, 24, 48 and 72 h [C+N+P(4)]. A control without the addition of any substrate was included. Soil moisture was the same in the treatments and control.

Respiration rates were determined after 4, 24, 48, 72, 96, 168 and 264 h (0.167, 1, 2, 3, 4, 7 and 11 days) by trapping CO_2 in 1 M NaOH. The residual NaOH was titrated with 0.1 M HCl after carbonates were precipitated with 0.5 M $BaCl_2$. Aliquots of the soil were removed, ATP was extracted immediately and the remaining aliquot was stored at –21°C prior to the determinations of the enzymatic activities.

Soil ATP content was measured according to Jenkinson and Oades (1979) by using 0.5 M trichloroacetic acid (TCA)/0.25 M disodium hydrogen phosphate (without paraquat) as extractant and

by sonicating soil suspensions for 2 min (about 60% of 100 W). Results were corrected for the percentage recovery of ATP; the ATP spike was 151.3 pg ATP g^{-1} soil.

β-1,4-glucosidase activity (E.C. 3.2.1.21) was determined according to Hoffmann and Dedeken (1965). Fresh soil (5 g) was mixed with 5 ml of 2 M sodium acetate buffer (pH 6.2) and 5 ml of 70 mM salicin [2-*O*-(β-D-glucopyranosido)-benzylalcohol; Merck 7665]. In the controls, 5 ml deionised water was used instead of the salicin solution. After incubating for 3 h at 37°C, samples were filtered (Schleicher and Schuell no 512; Schleicher and Schuell, Dassel, Germany); 3 ml of the filtrate was transferred to a 50-ml volumetric flask and then mixed with 5 ml of 200 mM borate buffer (pH 10.0) and 0.5 ml of 6.68 mM 2,6-dibromoquinone-4-chlorimide (Merck 13125). After 1 h, the volume was brought to 50 ml and the absorbance was measured at 578 nm. Standards were prepared with 0, 1, 2, 5, 10 and 15 ml of an 80.5 μmol phenol solution (7.58 mg phenol l^{-1}).

Protease activity was estimated according to Ladd and Butler (1972). Fresh soil (1 g) was mixed with 5 ml of 50 mM TRIS(hydroxymethyl)amino-methane buffer (pH 8.1) and 5 ml of 2% (w/v) casein (Sigma C 8654; suspended in this buffer). The soil mixture was incubated in a water bath at 50°C for 2 h; the substrate suspension was added to the controls after incubation. The reaction was stopped with 5 ml of 918 mM TCA and then the suspension was filtered (Schleicher and Schuell no. 595). Concentrations of released amino acids were measured photometrically by the Lowry method. Standards were prepared with 0, 0.4, 1.0, 2, 4 and 6 ml of a 0.05% (w/v) tyrosine solution (2.76 mM) made up to 10 ml with buffer and treated with TCA as reported above.

Urease activity was measured according to Kandeler and Gerber (1988). Fresh soil (2 g) was incubated for 2 h at 37°C with 1 ml of 0.08 M urea solution. In the controls, 1 ml deionised water was used instead of the urea solution. After incubation, 1 ml deionised water and 1 ml urea solution were added to the substrate-supplemented and control samples, respectively. Then, the produced NH_4^+ was extracted by adding 8 ml of 2 M KCl; the soil mixture was shaken for 15 min and centrifuged for 10 min at 3,000 r.p.m. The supernatant (0.5 ml) was treated with 4.5 ml deionised water, 0.2 ml of a 6.71 mM nitroprusside sodium dihydrate/1.062 M sodium salicylate solution and 0.2 ml of a 4:1-mixture (v v^{-1}) of 0.68 M trisodium citrate/0.5 M NaOH and 22.66 mM dichloroisocyranuric acid sodium salt dihydrate. After 90 min, the absorbance was determined at 690 nm. For the preparation of standards, 0, 0.25, 0.5, 1, 2.5, 4, 6, 8 and 10 ml of a 100-fold diluted 71.4 mM ammonium chloride solution were made up to 10 ml using 2 M KCl and then the NH_4^+ concentration was determined in aliquots of 0.5 ml as reported above.

Phosphatase activity was determined according to Hoffmann (1968). Fresh soil (5 g) was incubated for 3 h at 37°C in 10 ml of 0.2 M borate buffer (pH 10.0) and 5 ml of 0.1 M diphenyl phosphate disodium. In the controls, deionised (5 ml) water was used instead of the PO_4^{3-} solution. Following filtration (Schleicher and Schuell no. 512), 0.125 ml filtrate was transferred to a 50-ml volumetric flask containing 5 ml borate buffer and then 0.5 ml of 6.68 M 2,6-dibromo-quinone-4-chlorimide was added. After 30 min, the volume was brought to 50 ml with deionised water and absorbance measured at 578 nm. Standards were prepared with 0, 2.5, 5, 7.5 and 10 ml of a 0.106 mM phenol solution. Phosphatase activity was determined without the buffer to reflect the real activity in soil (Dilly 1999; Schinner et al. 1996; Tabatabai and Bremner 1969).

Sulphatase activity was estimated according to Tabatabai and Bremner (1970). Fresh soil (0.5 g) was incubated for 1 h at 37°C with 2 ml of 0.5 M acetate buffer (pH 5.8) and 0.5 ml of 0.02 M potassium-*p*-nitrophenylsulphate (dissolved in the acetate buffer). After incubation, the reaction was stopped by adding 12.5 ml deionised water; the soil suspension was shaken for 10 min and filtered (Schleicher and Schuell no. 512); 6 ml filtrate and 4 ml of 0.5 M NaOH were mixed and the absorbance measured at 420 nm. Standards were prepared by using 2.5 ml of 0, 4, 8, 12, 16 and 20 μg *p*-nitrophenol ml^{-1} solutions, and were treated in the same way as samples after incubation.

Three independent replicates corresponding to the three different sampling locations were used for all measurements. Excel 5.0 (Microsoft) and SigmaStat (Jandel Scientific, Erkrath, Germany) were used for statistical procedures. Analyses were performed with 95% confidence limits. Two-way repeated measures ANOVA (two factor repetition) was applied to achieve the SE of the least significant mean. A Spearman rank order correlation analysis was performed because the normality test failed.

Results and discussion

Soil respiration and ATP content

The application of C, C+N, and C+N+P increased the microbial respiration rate in the arable and the forest soil in comparison to the control (Fig. 1). When the C+N and C+N+P additions were split, the cumulative microbial respiration was highest at the end of the incubation period. The differences in the cumulative respiration were small but mostly significant among treatments of the same soil. Indeed the respired C (calculated by subtracting the amount respired by the treated soil minus that of the control) ranged from 35% to 38% of the added glucose-C in the arable soil at the end of the incubation period. The range was 25–31% of the glucose-C in the treated forest soil samples. The higher proportion of respired glucose-C in the treated arable soil samples and the higher ATP increase compared to the initial ATP level (Fig. 1) may be indicative of a better efficiency of soil microbiota in utilising glucose (Dilly 2001; Dilly and Munch 1998), and was probably related to the structural composition of microbial biomass, e.g. a higher glucose-responsive biomass and bacteria-to-fungi ratio in the arable than in the forest soil (unpublished data).

During the first days of the experiment, the respiration rates in the arable soil were more drastically affected by N and P addition than in the beech forest soil (Fig. 1), although the native inorganic N content did not differ between the two soils and the P content was lower in the forest soil (Dilly 1999). Since the respiration rate of glucose-treated soils is affected by the availability of nutrients (Stotzky and Norman 1961), it may be hypothesised that the N and P pools available to microorganisms were lower in the arable than in the forest soil. Indeed the N and P compounds available to soil microbiota include not only the exchangeable NH_4^+-N and NO_3^--N but also low molecular weight organic N compounds, which can be taken up by soil microorganisms (Barraclough 1997), or mineralised to the inorganic forms.

The maximum ATP values were reached after 3 days in both the arable and forest soil when C+N or C+N+P were added in a single dose or split into four equal doses, and they preceded the maximum respiration rate (Fig. 1). This contradicts observations of Nannipieri et al (1978), who found respiration rates to reach a maximum before that of the ATP content in two Canadian soils amended with glucose and NO_3^-. The maximum ATP values were higher when substrates were added in a single dose. It is worth noting that the substrate addition

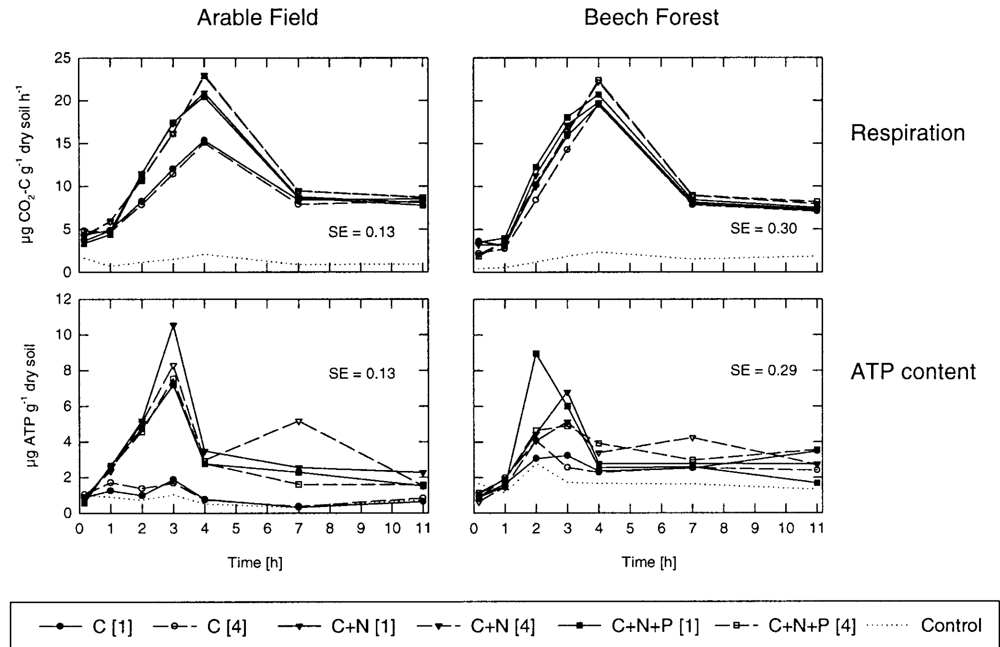

Fig. 1 Respiration rates and ATP content after the addition of glucose-C without (*C*) or with N or N+P applied at a single dose [1] or split into four equal doses over 4 consecutive days [4] in an arable and a forest soil. *SE* standard error of least significant mean

decreased ATP contents after 4 h, particularly when applied as a single dose. Osmotic effects due to the addition of nutrients to soil may have been responsible for the decrease in microbial activity. However, contradictory results have been reported in the literature. West and Sparling (1986) observed a decrease in CO_2 evolution in the early phase when glucose-C addition exceeded 24 mg ml^{-1} soil water. Recently, Lin and Brookes (2000) did not observe any inhibition of microbial respiration for glucose-C concentrations ranging from 0.17 to 28.4 mg ml^{-1} soil water. The glucose-C concentrations in the arable and the beech forest soil ranged from 19.4 to 19.7 and from 10.0 to 12.1 mg ml^{-1} soil water, respectively, for the single doses and, thus, they were in the range of the non-injurious concentrations studied by Lin and Brookes (2000).

Enzyme activities

β-Glucosidases often catalyse the rate-limiting reaction in the degradation of cellulose and are widely distributed among microorganisms and plants (Gong and Tsao 1979). Substrate addition initially inhibited ß-glucosidase activity in the two soils, and the inhibition was less pronounced in the beech forest than in the arable soil where it lasted for 3–4 days (Fig. 2). This response confirms the results of Chrost (1990), Carreiro et al. (1999) and Shackle et al. (2000), who showed that the presence of readily available glucose-C inhibits β-glucosidase activity. Furthermore, several salts including KNO_3 and KH_2PO_4 (at 8 mM concentration) have been found to inhibit β-glucosidase activity in soil, and the extent of inhibition depended on the type of soil (Eizavi and Tabatabai 1990). The β-glucosidase activity of the C+N+P[1]-treated arable soil was the lowest during 0–2 days of incubation, and then increased and reached the highest value at 7 days (Fig. 1). The β-glucosidase activity of the C+N- and C+N+P-treated forest soils increased in the 1- to 3-day incubation period (Fig. 2). Since both constitutive and inducible (by Avicel and cellobiose) enzymes have been detected in microbial cells (Wararchuk et al. 1984), N and P fertilisation may have increased the activity of microorganisms degrading cellulose either by promoting the growth of microbial species with constitutive enzymes or by stimulating enzyme synthesis in microbial cells with low enzyme activity under nutrient-deficient conditions.

The C(1) and C(4) treatments of the forest soil showed the highest casein-hydrolysing activities at the beginning of the incubation period (Fig. 2). In the two soils, the C+N+P(4) treatment generally decreased the casein-hydrolysing activity, except in the arable soil after 11 days (Fig. 2). Protease activity is controlled by nutrient conditions, is repressed by its catabolites (Kalisz 1988), and usually increases under conditions of starvation. This means that protease production is regulated by de-repression through the limitation of C, N and S sources and the absence of inducers. It may be hypothesised that the addition of C only caused starvation with an increase in enzyme activity, and the C+N+P[4] treatment offered available nutrients to soil microorganisms repressing protease synthesis. However, the C+N+P[4] treatment did not halt enzyme activity, probably because constitutive proteases were present in the two soils and/or some extracellular proteases were stabilised by soil colloids. No remarkable increase was observed in casein-hydrolysing activities immediately after the microbial growth indicated by the increasing ATP content (Fig. 1). Increases in casein-hydrolysing activities were

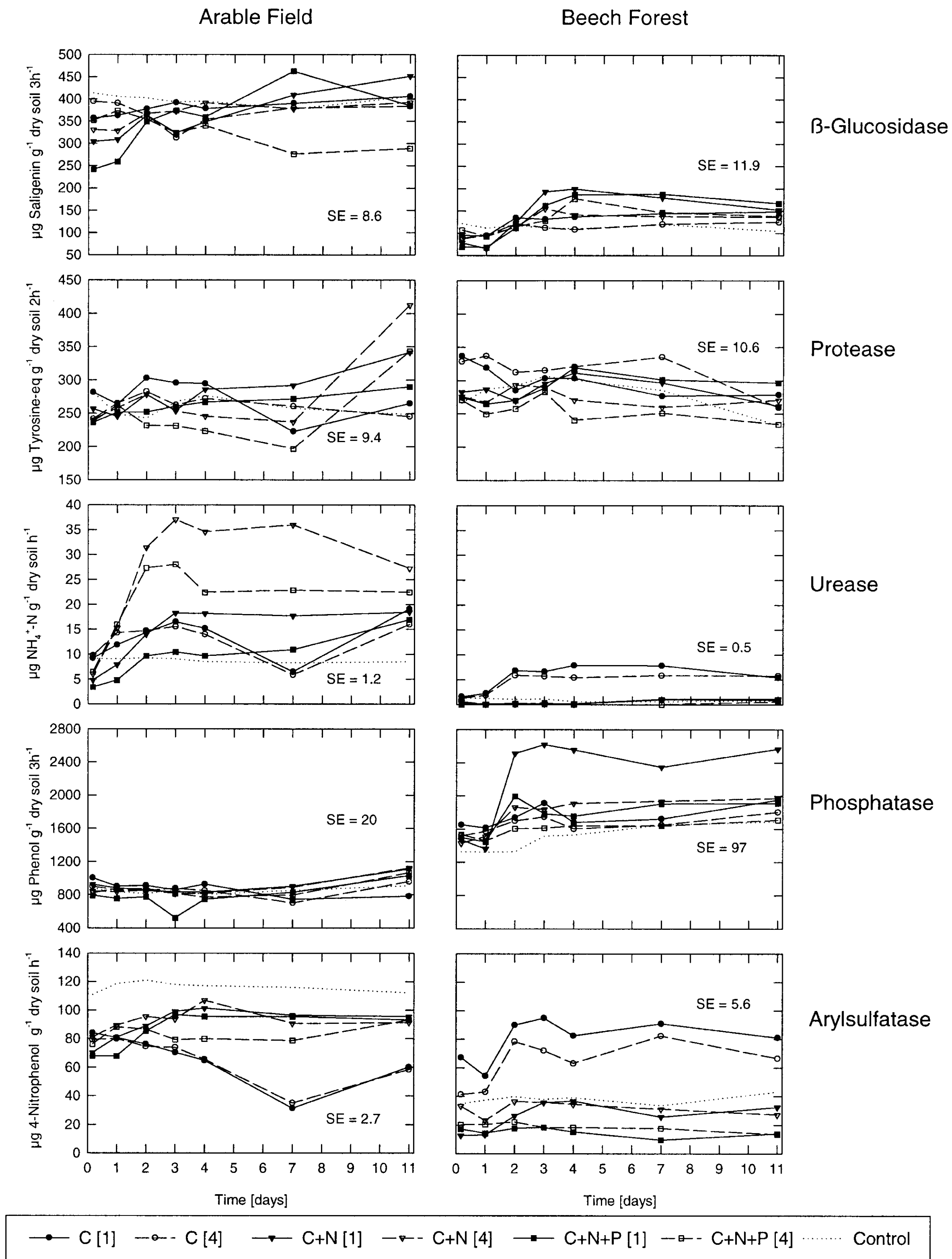

Fig. 2 Enzymatic activities after the addition of C[1], C[4], C+N[1], C+N[4], C+N+P[1] or C+N+P[4] in an arable and a forest soil. For abbreviations, see Fig. 1

late events, after the peaks in microbial biomass, phosphatase and urease activity in both C+N- and ryegrass+N-treated soils (Ladd and Paul 1973; Nannipieri et al. 1983). After microbial death and lysis, proteins were probably released into the environment, and the synthesis of proteases in the surviving microbial cells was induced. Correspondingly, Kandeler et al. (1999) have found that casein-hydrolysing activities are high in soil particles with a high microbial turnover.

Urease activity was inhibited by additions of C+N and C+N+P in the arable soil at day 0. Such an inhibition was not observed in the forest soil. The urease ac-

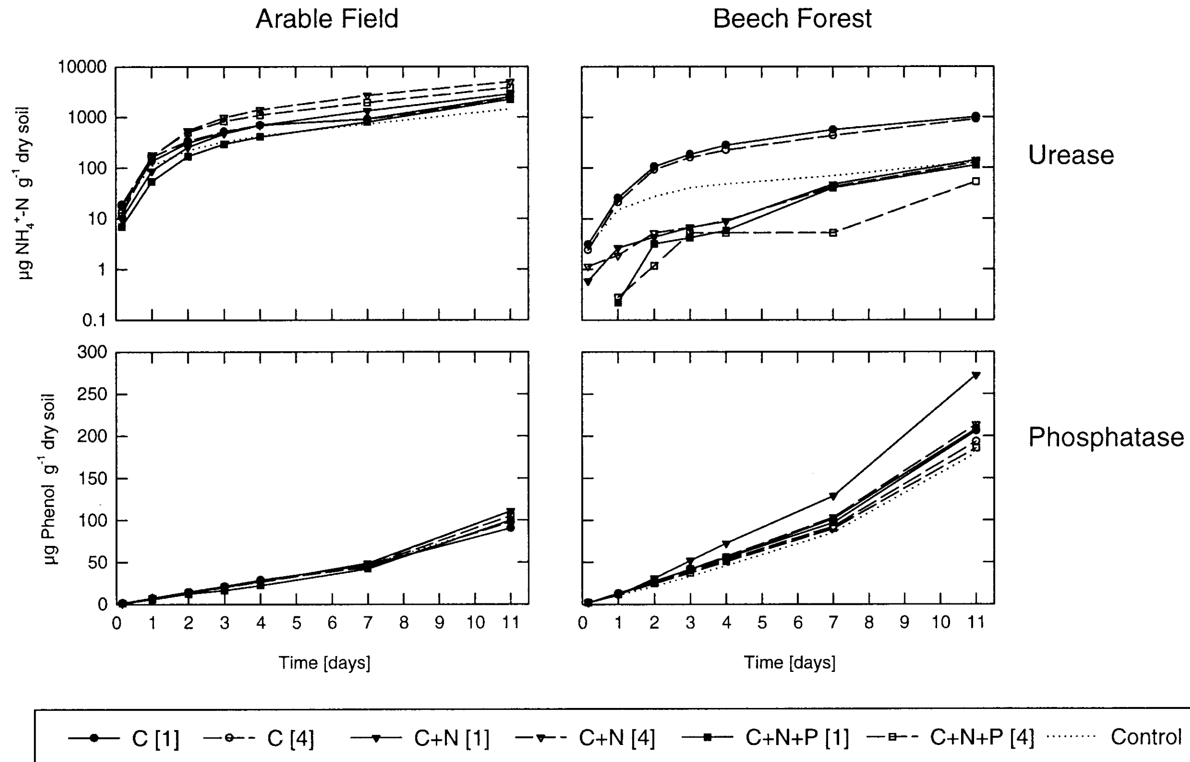

Fig. 3 Cumulative urease and phosphatase activity after the addition of C[1], C[4], C+N[1], C+N[4], C+N+P[1] or C+N+P[4] in an arable and a forest soil; activities displayed in Fig. 2 were multiplied by time before the measurements and added to the previous value. For abbreviations, see Fig. 1

tivity of the arable soil showed the highest increases when compared with other measured enzyme activities, and these increases were at their highest when nutrients were added in four doses (C+N[4] and C+N+P[4] treatments) (Fig. 2). On the other hand, the urease activity of the beech forest soil was only increased by the addition of C alone; N and P amendment repressed urease synthesis. The native urease level was lower in the beech than in the arable topsoil, although total microbial biomass estimated by the fumigation-extraction method was higher in the beech than in the arable soil (Dilly 1999). We, therefore, concluded that ureolytic microorganisms in the arable soil were more abundant and were more stimulated by C+N and C+N+P addition at small doses. However, ureolytic microorganisms need to be measured in order to verify the validity of this hypothesis, since constitutive and repressible ureases may have been present in the two soils. In pure culture the synthesis of repressible ureases depends on the amount and form of N compounds and on the microbial species (Mobley and Hausinger 1989). McCarty et al. (1992) showed that in glucose-amended soils urease activity decreased when NH_4^+ and NO_3^- concentrations increased. Furthermore, the repression of urease synthesis depended on the N products derived by microbial uptake of the inorganic forms, e.g. L- but not D-isomers of alanine, arginine, asparagine, aspartate and glutamine repressed urease production in glucose-amended soil (McCarty et al. 1992).

Unbuffered phosphatase activity in the arable soil fluctuated through the incubation period, but no remarkable increases were observed when C with or without N and P was added (Fig. 2). This activity even decreased when P was added together with C and N in a single dose (C+N+P[1]). PO_4^{3-} addition might have inhibited enzyme activity and also partially repressed microbial synthesis of the phosphatases. Indeed, microbial phosphatases are generally repressible enzymes (Hollander 1971). As already discussed by Nannipieri et al. (1978, 1990), most of the phosphatases contributing to the overall enzyme activity of soil are not repressed by P addition since they are stabilised by their interaction with soil colloids, or are constitutive and insensitive to P additions. The marked increase in phosphatase activity in the beech forest in response to the C+N[1] treatment was probably due to the low level of available P in this soil (Dilly 1999). Probably P addition in the C+N+P[1] treatment repressed the increase in the phosphatase activity (Figs. 2, 3). Treatments C(4) and C+N(4) did not enhance the enzyme activity, probably because these treatments stimulated only weak microbial growth, as evidenced by the behaviour of ATP (Fig. 1).

The arylsulphatase activity was generally reduced in the arable topsoil by nutrient addition while it was enhanced in the beech soil by the addition of C only. Glucose addition decreased sulphatase activity in English grassland soils (Shackle et al. 2000). According to Dodgson et al. (1982), sulphates, sulphides and phosphates act as non-competitive inhibitors of arylsulphatase, which is not a constitutive enzyme as its synthesis

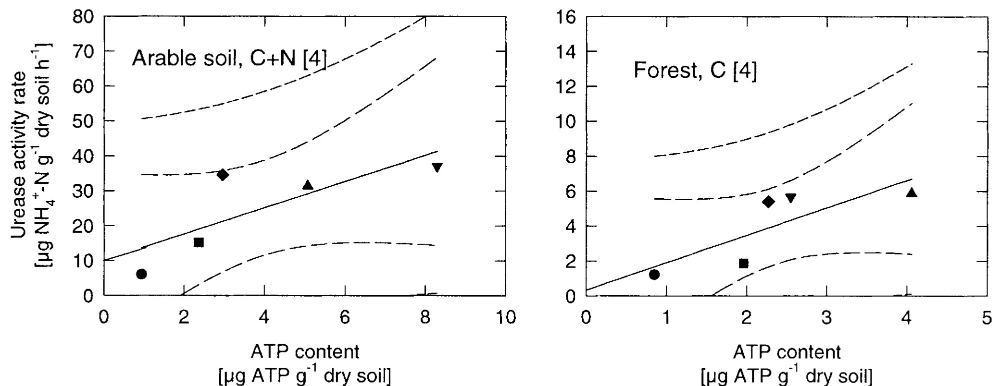

Fig. 4 Correlation between urease activity and ATP content in an arable soil treated with C+N[4] and a forest soil treated with C[4]. *Dashed lines* represent 95% and 99% confidence limits. For abbreviations, see Fig. 1

controlled by the C and S conditions. Tabatabai and Bremner (1970) found that phosphate, sulphite and cyanide inhibited arylsulphatase activity in soil, whereas sulphate and chlorides had little effect. Since soil S concentrations were not limiting microbial growth (Wellbrock, personal communication), the addition of C only to the beech forest soil probably increased the microbial requirement for S, leading either to the de-repression of enzyme synthesis or to the reduction of concentrations of SO_4^{2-} which acts as an enzyme inhibitor.

Correlation between enzyme activity and ATP content

According to the physiological approach, the extracellular enzyme activity can be determined if the correlation between the enzyme activity and microbial biomass is significant. Then, by plotting the enzyme activity against the microbial biomass, the extrapolation to zero biomass has to produce a positive intercept representing the extracellular enzyme activity (Nannipieri et al. 1996b, 2000; Dilly and Nannipieri 1998). Spearman rank order correlation coefficients between urease activity and ATP content in the arable soil were 0.93 (n=7) for C+N(4), and 1.00 (n=5; for the first five samplings) for C+N+P(4); in the forest soil, they were 0.89 (n=7) for C(4) and 1.00 (n=5; for the first five samplings) for C(4). Figure 4 shows the positive intercept for the two soils when fitting the data to a linear line. The intercept gave an urease activity of about 10 µg NH_4^+-N g^{-1} soil h^{-1} for the arable soil and <1 µg NH_4^+-N g^{-1} soil h^{-1} for the beech forest soil. The intercept and thus the extracellular urease activity in Canadian Bradwell grassland soil was about 5 µg N NH_4^+-N g^{-1} soil h^{-1} (Nannipieri et al. 1978). Although higher organic matter content and finer texture were present in the beech forest soil (Dilly and Nannipieri 1998), the stabilised extracellular urease activity in this soil was lower than that in the arable soil. This may be related to the stabilising effect of humic properties on extracellular ureases which are incorporated into the humic network (Nannipieri et al. 1996a).

β-Glucosidase, casein-hydrolysing protease, unbuffered phosphatase and arylsulphatase activities were not correlated to the ATP content during the time course of the experiment. Therefore, the approach used by McLaren and Pukite (1973) and Nannipieri et al. (1996b) to determine the extracellular enzyme activity in soil was not successful for these enzymes in the arable and forest soil. However, the ATP content may also not fully reflect changes in microbial biomass because it represents microbial activity when measured immediately after sampling (Nannipieri et al. 1990). On the other hand, the chloroform fumigation-extraction method cannot be used within the first few days after substrate additions because in the presence of high C contents the proportionality between chloroform-labile C and biomass C is not valid (Bremer and von Kessel 1990; Witter and Dahlin 1995). Marstrop and Witter (1999) have found that the quantitative determination of dsDNA in soil extracts accurately estimates microbial growth immediately after glucose addition to soil.

The glucose and nutrients were applied in doses typical for soil microbial assays (Alef and Nannipieri 1995) to support the rapid reproduction of glucose-utilising soil microorganisms (Bremer and von Kessel 1990). Glucose and NO_3^- at similar concentrations supported microbial growth and the increase in phosphatase activity over 96 h (Nannipieri et al. 1996b). In contrast, Nannipieri et al. (1978) found that microbial growth was short-lived with similar applications since the ATP content and phosphatase activity decreased after 48 h. Since substrate dosages were high in comparison to levels present under field conditions, substrates applied at low rates over extended periods may support continuous enzyme production and additionally enable the use of the fumigation-extraction method for the estimation of microbial biomass.

In conclusion, the main findings of the work and future research needs can be summarised in the following way:

1. The response of the ATP content and enzyme activities to nutrient addition depended on both enzymes and soil. The arable and forest soil seem to differ in microbial structure and ecophysiology. Indeed, differences in their bacterial-fungal ratios and proportion of r vs. K strategists have been reported (Dilly 1996; Dilly and Munch 1998).
2. The proposed method for determining the extracellular contribution to the overall activity of an enzyme

in soil may not be suitable for repressible and inducible enzymes. Thus, changes in enzyme activity can only be associated with microbial growth in the absence of compounds repressing or inducing enzyme synthesis.

3. The data obtained in this work also underline the need for further research by using a better method for determining microbial biomass. McLaren and Pukite (1973) correlated urease activity and ureolytic microorganisms, indicating that we probably need to adopt for each enzyme an assessment of the appropriate component of the biomass.
4. The physiological method for the determination of the extracellular enzyme activity is conceptually different from the chloroform-fumigation method proposed by Klose and Tabatabai (1999). The enzyme activity determined before and after soil fumigation is due to extracellular enzyme activity, and both intracellular and extracellular enzyme activity, respectively (Klose et al. 1999). In contrast, the physiological method assumes that the current enzyme assays determine the contribution of both intracellular and extracellular enzyme activities in soil. The fact that some enzyme activities were correlated with microbial growth seems to support the hypothesis that some of the measured enzyme activity was intracellular.

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References

Alef K, Nannipieri P (1995) Methods in applied soil microbiology and biochemistry. Academic Press, London

Anderson JPE, Domsch K-H (1973) Quantification of bacterial and fungal contribution to soil respiration. Arch Mikrobiol 93:113–127

Barja I, Nunez L (1999) Microcalorimetric measurements of the influence of glucose concentration on microbial activity in soil. Soil Biol Biochem 31:441–447

Barraclough D (1997) The direct or MIT route for nitrogen immobilization: a ^{15}N mirror image study with leucine and glycine. Soil Biol Biochem 29:101–108

Bremer E, Kessel C von (1990) Extractability of microbial ^{14}C and ^{15}N following addition of variable rates of glucose and $(NH_4)_2SO_4$ to soil. Soil Biol Biochem 22:707–713

Burns RG (1978) Soil enzymes. Academic Press, London

Burns RG (1982) Enzyme activity in soil: location and a possible role in microbial ecology. Soil Biol Biochem 14:423–427

Carreiro M, Sinsabaugh R, Repert D, Pankhurst D (1999) Shifts in microbial enzymes and forest litter decay in response to nitrogen deposition. In: Dick RP (ed) Enzymes in the environment: activity, ecology and application. CSIC, Granada, Spain, p 65

Chrost RJ (1990) Microbial ectoenzymes in aquatic environments. In: Overbeck J, Chrost RJ (eds) Aquatic microbial ecology. Biochemical and molecular approaches. Springer, Berlin Heidelberg New York, pp 47–78

Dilly O (1996) Charakterisierung mikrobieller Populationen in Böden durch Methoden zur Quantifizierung der mikrobiellen Biomasse. Mitt Dtsch Bodenkd Ges 81:369–372

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

Dilly O (2001) Metabolic and anabolic responses of four arable and forest soils to nutrient addition. Z Pflanzenernähr Bodenkd 164:29–34

Dilly O, Munch JC (1998) Ratios between estimates of microbial biomass content and microbial activity in soils. Biol Fertil Soils 27:374–379

Dilly O, Nannipieri P (1998) Intracellular und extracellular enzyme activity in soil with reference to elemental cycling. Z Pflanzenernähr Bodenkd 161:243–248

Dodgson KS, White GF, Fitzgerald JW (1982) Sulfatases of microbial origin, vol II. CRC Press, Boca Raton, Fla.

Drobnik J (1960) Primary oxidation of organic matter in the soil. I. The form of respiration curves with glucose as the substrate. Plant Soil 12:199–211

Eizavi F, Tabatabai MA (1990) Factors affecting glucosidase and galactosidase activities in soil. Soil Biol Biochem 22:891–897

FAO (1988) Soil map of the world. Revised legend. World soil resources report no. 60. FAO, Rome

Friedel JK, Munch JC, Fischer WR (1996) Soil microbial properties and the assessment of available soil organic matter in a haplic Luvisol after several years of different cultivation and crop rotation. Soil Biol Biochem 28:479–488

Gong CS, Tsao GT (1979) Cellulase and biosynthesis regulation. Annu Rep Ferm Proc 3:111–140

Hoffmann G (1968) Eine photometrische Methode zur Bestimmung der Phosphatase-Aktivität in Böden. Z Pflanzenernähr Bodenkd 118:161–172

Hoffmann G, Dedeken M (1965) Eine Methode zur colorimetrischen Bestimmung der β-Glucosidase-Aktivität in Böden. Z Pflanzenernähr Dueng Bodenkd 108:193–198

Hollander VP (1971) Acid phosphatases. In: Boyer PD (ed) The enzyme. Academic Press, New York, pp 449–498

Jenkinson DS, Oades JM (1979) A method for measuring adenosine triphosphate in soil. Soil Biol Biochem 11:193–199

Kalisz HM (1988) Microbial proteinases. Adv Biochem Eng Biotechnol 36:3–60

Kandeler E, Gerber H (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fertil Soils 6:68–72

Kandeler E, Luxhoi J, Tscherko D, Magid J (1999) Xylanase, invertase and protease at the soil-litter interface of a loamy sand. Soil Biol Biochem 31:1171–1179

Klose S, Tabatabai MA (1999) Urease activity of microbial biomass in soils. Soil Biol Biochem 31:205–211

Klose S, Moore JM, Tabatabai MA (1999) Arylsulfatase activity of microbial biomass in soils as affected by cropping system. Biol Fertil Soils 29:46–54

Ladd JN, Butler JHA (1972) Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivates as substrates. Soil Biol Biochem 4:19–30

Ladd JN, Paul EA (1973) Changes in enzyme activity and distribution of acid-soluble amino acid-nitrogen in soil during nitrogen immobilization and mineralization. Soil Biol Biochem 5:825–840

Lin Q, Brookes PC (2000) An evaluation of the substrate-induced respiration method. Soil Biol Biochem 31:1969–1983

Marstrop H, Witter E (1999) Extractable dsDNA and product formation as measures of microbial growth in soil upon substrate addition. Soil Biol Biochem 31:1443–1453

McCarty GW, Shogren DR, Bremner JM (1992) Regulation of urease production in soil by microbial assimilation of nitrogen. Soil Biol Biochem 12:261–264

McLaren AD, Pukite A (1973) Ubiquity of some soil enzymes and isolation of soil organic matter with urease activity. In: Povoledo D, Golterman ML (eds) Humic substances and function in the biosphere. Pudoc, Wageningen, The Netherlands, pp 187–193

Mobley HLT, Hausinger RP (1989) Microbial urease: significance, regulation, and molecular characterization. Microbiol Rev 53:85–108

Nannipieri P, Johnson RL, Paul EA (1978) Criteria for measurement of microbial growth and activity in soils. Soil Biol Biochem 10:223–229

Nannipieri P, Pedrazzini F, Arcara PG, Piovanelli C (1979) Changes in amino acids, enzyme activities and biomasses during microbial growth. Soil Sci 127:26–34

Nannipieri P, Muccini L, Ciardi C (1983) Microbial biomass and enzyme activity: production and persistence. Soil Biol Biochem 15:679–685

Nannipieri P, Ceccanti B, Grego S (1990) Ecological significance of the biological activity in soil. In: Bollag J-M, Stotzky G (eds) Soil biochemistry, vol 6. Dekker, New York, pp 293–355

Nannipieri P, Sequi P, Fusi P (1996a) Humus and enzyme activity. In: Piccolo A (ed) Humic substances in enzyme activity. Elsevier, Amsterdam, pp 293–328

Nannipieri P, Sastre I, Landi L, Lobo MC, Pietramellara G (1996b) Determination of extracellular neutral phosphomonoesterase activity in soil. Soil Biol Biochem 28:107–112

Nannipieri P, Kandeler E, Ruggiero P (2000) Enzymes activities as indicators of soil microbial functional diversity. In: Dick R (ed) Enzymes in the environment: activity, ecology and applications. CSIC, Granada, Spain, p 13

Schinner F, Öhlinger R, Kandeler E, Margesin R (1996) Methods in soil biology. Springer, Berlin Heidelberg New York

Shackle VJ, Freeman C, Reynolds B (2000) Carbon supply and the regulation of enzyme activity in constructed wetlands. Soil Biol Biochem 32:1935–1940

Skujins J (1978) History of abiontic soils enzyme research. In: Burns RG (ed) Soil enzymes. Academic Press, London, pp 1–49

Sparling GP (1995) The substrate-induced respiration method. In: Alef K, Nannipieri P (eds) Methods in applied soil microbiology and biochemistry. Academic Press, London, pp 397–404

Stotzky G (1965) Microbial respiration. In: Black CA (ed) Methods of soil analysis. American Society of Agronomy, Madison, Wis., pp 1550–1572

Stotzky G, Normann AG (1961) Factors limiting microbial activities in soil. 1. The level of substrate, nitrogen and phosphorus. Arch Mikrobiol 40:341–369

Tabatabai MA, Bremner JM (1969) Use of *p*-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol Biochem 1:301–307

Tabatabai MA Bremner JM (1970) Arylsulfatase activity of soils. Soil Sci Soc Am Proc 34:225–229

Wararchuk WW, Kilburn DG, Miller RC Jr, Warren RAJ (1984) The preliminary characterisation of the β-glucosidase of *Cellulomonas fimi*. J Gen Microbiol 130:1385–1389

West AW, Sparling GP (1986) Modification to the substrate-induced respiration method to permit measurement of microbial biomass in soils of differing water contents. J Microbiol Methods 5:177–189

Witter E, Dahlin S (1995) Microbial utilization of (U-^{14}C)-labelled straw and (U-^{13}C)-labelled glucose in soil of contrasting pH and metal status. Soil Biol Biochem 27:1507–1516