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Hydrolase activities during and after the chloroform fumigation of soil as affected by protease activity

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Abstract

$CHCl_3$ fumigation was used to induce changes in hydrolase activities of a Vertisol under different management (woodland, grassland and arable soils). Measurements were made during the standard 24 h $CHCl_3$ fumigation period used for biomass measurement and after incubation following fumigation. Fumigation of soil was very effective in killing microorganisms as shown by the marked decrease in the ATP content after 4 h of $CHCl_3$ exposure. However, it did not change or slightly decreased β-glucosidase, acid phosphomonoesterase, alkaline phosphomonoesterase and protease as measured by hydrolysis of *N*-benzoylargininamide, markedly decreased urease activity and increased arylsulphatase activity.

$CHCl_3$ fumigation of soils for 24 h in the presence of several protease inhibitors decreased *N*-benzoylargininamide hydrolising activities by more than 50% and increased urease and phosphomonoesterase activities, demonstrating that enzymes released during cell lyses can undergo to proteolysis during the $CHCl_3$ fumigation.

During the incubation following fumigation, respiration increased following the typical trend. ATP content also increased in all soils because of the growth of the small microbial population surviving the fumigation. During the incubation following fumigation, β-glucosidase and phosphomonoesterase activities recovered to values not significantly different from those of unfumigated and incubated soil. Arylsulphatase activity decreased in all fumigated soils reaching values not significantly different from those of the unfumigated soils after 24 h. Benzoylargininamide hydrolyzing activities showed only minor changes during the incubation of unfumigated and fumigated soils. Urease activity decreased to low levels in the woodland soil and was not measurable in the grassland and arable soils. N metabolites, derived from the microbial NH_4^+ immobilization during the incubation following fumigation, may have inhibited the urease synthesis in microbial cells.

Keywords: Hydrolase activities; Intracellular enzyme activity; Extracellular enzyme activity; $CHCl_3$ fumigation; ATP content

1. Introduction

In the soil environment, active enzymes exist within the microbial cells, adsorbed onto soil colloids, free in soil solution and associated with cell debris (Burns, 1982). Active enzymes, present in the soil solution (free enzymes) or in cell debris, are short-lived and their contribution to the overall enzyme activity is considered to be negligible (Burns, 1982). The measurement of the extra- and intracellular enzyme activity in soil is needed for a better understanding of the response of enzyme activities to changes in agricultural practices, environmental conditions and presence of toxicants (Nannipieri, 1994). Intracellular enzyme activity may be related to microbial activity, while the activity of the soil-stabilised enzymes, protected from thermal and chemical denaturation (Perez-Mateos and Rad, 1989; Speir and Ross, 1990; Nannipieri, 1994) and proteolysis (Nannipieri, 1994), may be insensitive to such changes.

Chemical and physical microbial inhibitors have been used for measuring the contribution of the intracellular enzyme activity to overall soil enzyme activity. These approaches have produced conflicting results (Kiss et al., 1975; Burns, 1978; Ladd, 1985; Frankenberger and Johanson, 1986), possibly due to non-target effects, adsorption onto soil colloids and microbial degradation (Landi et al., 1993; Nannipieri, 1994; Nannipieri et al., 2001). In addition, toluene, a bacteriostatic agent, has been found to increase membrane permeability and thus intracellular enzyme activity (Skujins 1978).

A different approach was followed by McLaren and Pukite (1973) who correlated the number of ureolytic microorganisms to urease activity after microbial growth induced by carbon and nitrogen sources added to soils.

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Both microbial numbers and urease activity values were those reported by Paulson and Kurtz (1969). The significant correlation plot showed a positive intercept at the zero microbial biomass value, and the intercept value was assumed to be the extracellular enzyme activity. The same approach was followed by Nannipieri et al. (1996) to measure the extracellular phosphomonoesterase activity. They measured ATP content, which is a better measurement of microbial biomass than the plate count, which only reveals 1–10% of the total soil microbial population (Torsvik et al., 1996). Advantages and limitations of this approach, called the Physiological Approach, have been discussed by Nannipieri et al. (2001).

Recently, the $CHCl_3$ fumigation method used for estimating microbial biomass C, has been adapted to assess the intracellular urease and arylsulphatase activities, but not the intracellular activities of 10 other enzymes involved in the C, N, P and S cycling (Klose and Tabatabai, 1999a,b; Klose et al., 1999). After the fumigation treatment, both the arylsulphatase and urease activities were significantly higher (more than 50%) than those of the control soils. It was hypothesized that the increased activity was due to the release and measurement of the intracellular enzyme activities, whereas the activity of the unfumigated soils was assumed to be due to the extracellular and soil-stabilised enzymes. However, no data were reported on the behaviour of the urease and arylsulphatase activities during the 24-h fumigation period and no microbial parameters were monitored to determine the efficacy of $CHCl_3$ fumigation in lysing soil microorganisms.

The aims of this research were to study:

1. the effect of the $CHCl_3$ fumigation on β-glucosidase, arylsulphatase, urease, protease, acid and alkaline phosphomonoesterase activities in order to assess the validity of $CHCl_3$ fumigation for determining intracellular activities of these enzymes;
2. the effects of protease activity released by cell lysis on the persistence of hydrolases released during the 24-h fumigation period;
3. the trend of these enzyme activities during the early phases following $CHCl_3$ fumigation and their relation to microbial activity as determined by the CO_2 evolution.

The ATP content was used as an index of microbial biomass not only to assess the efficiency of $CHCl_3$ fumigation in lysing microbial cells, but also to follow the growth of surviving microorganisms in the incubation following fumigation.

The three soils differ in their microbial biomass, organic matter contents and enzyme activities (Table 1). Other soil properties (pH, soil texture and clay mineralogy) are similar so that any differences in cell lysis by $CHCl_3$ fumigation among the soils should be due to differences in organic matter content and microbial biomass. It has been reported that the efficiency of the $CHCl_3$ fumigation can be affected by several soil properties (Badalucco et al., 1997).

2. Materials and methods

2.1. Soils

The study was conducted on a calcareous soil, Vertic Xerochrept (USDA, 1992) of the Vicarello experimental area located in Tuscany (Central Italy), controlled by the Institute for Soil Study and Protection (Ministry of the Agricultural and Forest Policies). Soil samples were collected under three different permanent management regimes (wheat, grassland and woodland) and they differ only in organic C, microbial biomass content and hydrolase activities (Table 1). Soils were surface (0–10 cm for the woodland and grassland soils and 0–25 cm for the arable soil) sampled in January 2000, sieved (<2 mm) with removal of visible animals and plant residues by hand, moistened up to the 50% of the water holding capacity (WHC) and preincubated for 7 days prior to analyses and fumigation (Table 1).

2.2. Fumigation and incubation conditions

The soils were fumigated with ethanol-free $CHCl_3$ and after 4, 12 and 24 h the fumigant was removed and the enzyme activities and ATP content determined. Commercial jack-bean urease (from Sigma), and wheat germ acid phosphomonoesterase (from Fluka) were also fumigated as dry enzymes for 24 h to assess the effects of the fumigant on the activity of pure enzymes.

Unfumigated or fumigated (24 h) soils (25 g o.d) were incubated in 1-l sealed glass jars with separate vials containing water or 1 M NaOH (10 ml each). The excess of alkali was back titrated with standard 0.1 M HCl after precipitating the carbonate with 0.75 M $BaCl_2$ (Badalucco et al., 1997). Cumulative CO_2 evolution was measured after 10 days. Enzyme activities, ATP content and soil respiration were measured after 1, 4 and 10 days of incubation.

Protease activity was inhibited using an inhibitor cocktail (from SIGMA) designed for inactivating proteases of bacterial cell extracts. The cocktail contained the following inhibitors dissolved in the DMSO:water solution (1:5 ratio): 18 mM 4-(2-aminoethyl) benzenesulphonyl fluoride (AEBSF) (an inhibitor of exo- and endo-Ser-type proteases and aminopeptidases), 2.5 mM pepstatin A (an inhibitor of acid proteases including pepsin), 0.22 mM trans-epoxy-succinyl-L-leucyl-amido(4-guanidino)butane (E-64) (an inhibitor of cys-type proteases), 1.7 mM bestatin (an inhibitor of metalloproteases and aminopeptidases), and 8.6 mM sodium-EDTA (which chelates the metallic cations essential to metalloproteases). In addition to the concentration proposed by the supplier (the highest, as reported above) we also prepared another solution corresponding to a 10-fold dilution of that (the lowest). Five ml of inhibitor

Table 1
Some chemical, biochemical and microbiological characteristics of the Vicarello soils (TOC, total organic carbon; N_{tot}, total nitrogen; Alk Ph, alkaline phosphomonoesterase activity; Ac Ph, acid phosphomonoesterase activity; β-Gluc, β-glucosidase activity; ArS, arylsulphatase activity; protease, *N*-Benzoylargininamide (*N*-BAA) hydrolasing activity; Urease, urea amydohydrolase activity; *$P < 0.05$; **$P < 0.01$)

Soil/land use	$pH_{(H_2O)}$	TOC (%)	N_{tot} (%)	Microbial biomass C (μg g^{-1} soil)	ATP (ng g^{-1} soil)	Soil respiration (μg CO_2–C g^{-1} soil * h)	Alk Ph (μg *p*-NP g^{-1} soil * h)	Ac Ph (μg *p*-NP g^{-1} soil * h)	β-Gluc (μg *p*-NP g^{-1} soil * h)	ArS (μg *p*-NP g^{-1} soil * h)	Protease (μg $NH_4^+ - N\ g^{-1}$ soil * h)	Urease (μg $NH_4^+ - N\ g^{-1}$ soil * h)
Woodland	8.1	2.4*	0.22*	802**	3415**	3.27*	5327.9*	3476.8*	4669.0**	2902.2**	80.5**	35.9*
Grassland	8.1	1.8*	0.19*	518*	1462*	2.88	3694.6	987.6	1847.3	806.5	20.12*	9.9
Wheat	8.1	1.08	0.09	222	724	2.40	3298.9	695.6	1553.1	745.7	12.0	7.93

cocktail were added dropwise to 50 g (dry weight equivalent of soil). Then, each soil sample was split in two sub-samples (25 g dry weight each); one was immediately fumigated with ethanol-free $CHCl_3$ for 24 h and the other left at room temperature for the same period of time. The enzyme activities were measured on both the fumigated and unfumigated soils as described below.

2.3. Analytical measurements

β-glucosidase activity was determined as reported by Tabatabai (1982), arylsulphatase activity by Tabatabai and Bremner (1972), urease activity by Nannipieri et al. (1978), *N*-Benzoylargininamide (BAA) hydrolyzing activity by Ladd and Butler (1972), acid and alkaline phosphomonoesterase activities by Tabatabai and Bremner (1969). All enzyme assays (Table 1) were carried out at 37°C for 1 h, with centrifugation of soil slurries at $6000 \times g$ at 4°C. The concentration of p-nitrophenol (PNP) produced in the assays of β-glucosidase, arylsulphatase, acid and alkaline phosphomonoesterase activities was calculated from a PNP calibration curve after subtraction of the absorbance of the blank. Optical density was measured at 400nm wavelength. The NH_4^+ produced during the assays of urease and BAA hydrolasing activities was determined by a Flow Injection Analyzer (FIAS 300-Perkin Elmer) associated to a spectrophotometer Lambda 2 (Perkin-Elmer).

The ATP content was measured according to the method of Webster et al. (1984) as modified by Ciardi and Nannipieri (1990). The respiration of both fumigated and unfumigated soils was determined by back-titration of NaOH with standardised 1.0 M HCl as reported above.

All the presented data are the mean of three replications and expressed on the dry mass basis (24 h, 105°C).

2.4. Statistics

Analysis of variance was used (Tukey's test, $P < 0.05$) to assess the statistical significance of differences between the means.

3. Results and discussion

3.1. The behaviour of ATP and CO_2

The ATP content of soils (Fig. 1) decreased in all soils to very low values after 4 h of $CHCl_3$ fumigation, confirming that this treatment is effective in killing microorganisms. The decrease in the ATP content was more rapid than the decline observed by Arnebrant and Schnurer (1990). It has been hypothesised that ATP synthesis is inhibited whereas ATPases remain active during the $CHCl_3$ fumigation (Ciardi et al., 1993). Indeed, both acid and alkaline phosphomonoesterases were active during the fumigation of soils (Fig. 4).

After the removal of the fumigant, ATP values increased slightly in all soils indicating the persistence of a small

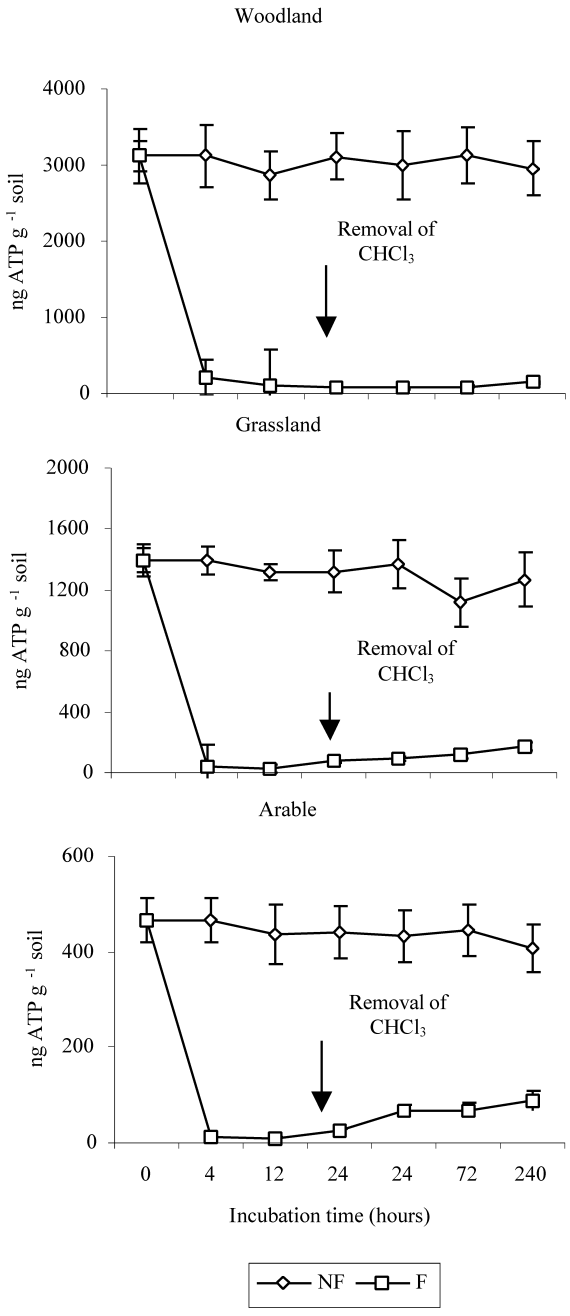

Fig. 1. ATP content of the soils during and after the 24 h $CHCl_3$ fumigation. It is also reported the values of the unfumigated soils incubated for the same period of time. The error bar is the standard error of the means ($n = 3$).

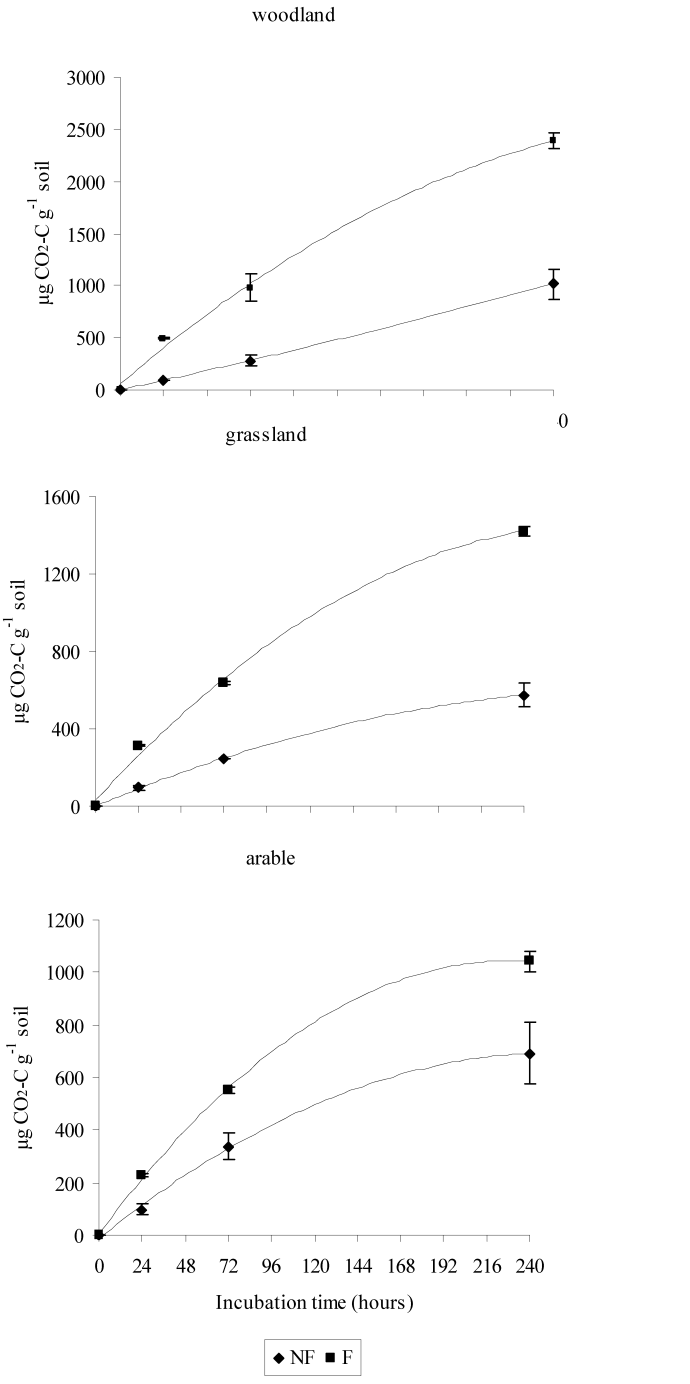

Fig. 2. Carbon dioxide evolution from the unfumigated and fumigated soils during incubation after the removal of $CHCl_3$. The error bar is the standard error of the means ($n = 3$).

microbial population. Similar behaviour has been reported by Lin and Brookes (1999). It is known that the $CHCl_3$ fumigation of soils does not kill all the microorganisms (Jenkinson, 1988) and that cell lysis depends on the composition of soil microbiota and the location of soil microorganisms in the soil structure. For example, Ingham and Horton (1987) and Ciardi et al. (1993) reported that both bacterial and fungal counts were slightly decreased by $CHCl_3$ fumigation of soils. The observation by transmission electron microscopy of soil ultrathin sections showed that bacteria located in small pores or embedded in the mucigel can survive the fumigation (Martin and Foster, 1985; Foster, 1988). For this reason, the efficiency of $CHCl_3$ fumigation in lysing cells depends on soil properties such as porosity and organic matter, clay and carbonate contents (Badalucco et al., 1997).

The CO_2–C evolution after the removal of the fumigant followed the typical trend (Fig. 2) already reported by

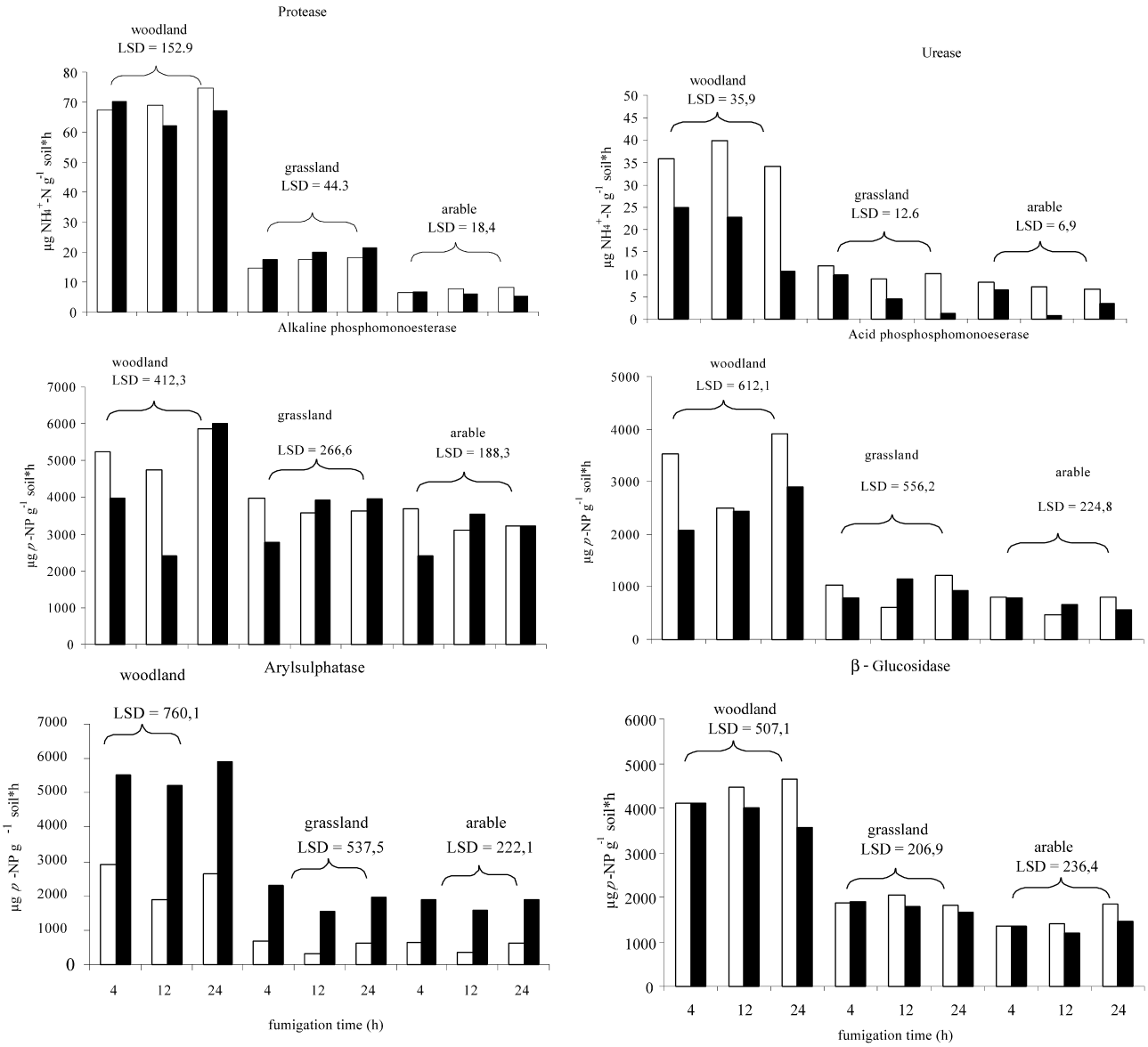

Fig. 3. The hydrolase activity during the course of the 24 h of $CHCl_3$ fumigation. Also reported are the values of the unfumigated soils. □, unfumigated; ■, fumigated.

Jenkinson and Powlson (1976). There was an initial flush of CO_2–C in the fumigated soils followed by a decrease to the same value as unfumigated soils. The initial CO_2–C flush depends on the decomposition of killed cells by the surviving microorganisms and it was supposed to be proportional to microbial biomass C content of soil before fumigation (Jenkinson and Powlson, 1976).

3.2. Enzyme activities

The activities of the pure urease, and acid phosphomonoesterase were not affected by 24 h of $CHCl_3$ fumigation (data not shown). $CHCl_3$ fumigation decreased by 29% purified urease and by 29 and 50% two purified (from *H. pomatia* and *P. vulgata*, respectively) arylsulphatase (Klose and Tabatabai, 1999a,b) activities. By considering activity values of soil and the percentage of enzyme protein in the preparation, urease and arylsulphatase proteins content of soil were calculated. These calculations were based on the assumption that the status of ureases and arylsulphatases in soil were similar to those of the references proteins used. However, the use of commercial enzymes is not a reliable control because these enzymes do not resemble those of soil. As reported in the introduction enzymes are present in soil in different locations and free enzymes being short-lived are supposed to give a scarce contribution to the overall enzyme activity (Burns, 1982; Nannipieri, 1994).

Generally, enzyme activities were significantly higher ($P < 0.05$) in the woodland soil and lower in the arable soil (Table 1), confirming that hydrolase activities are

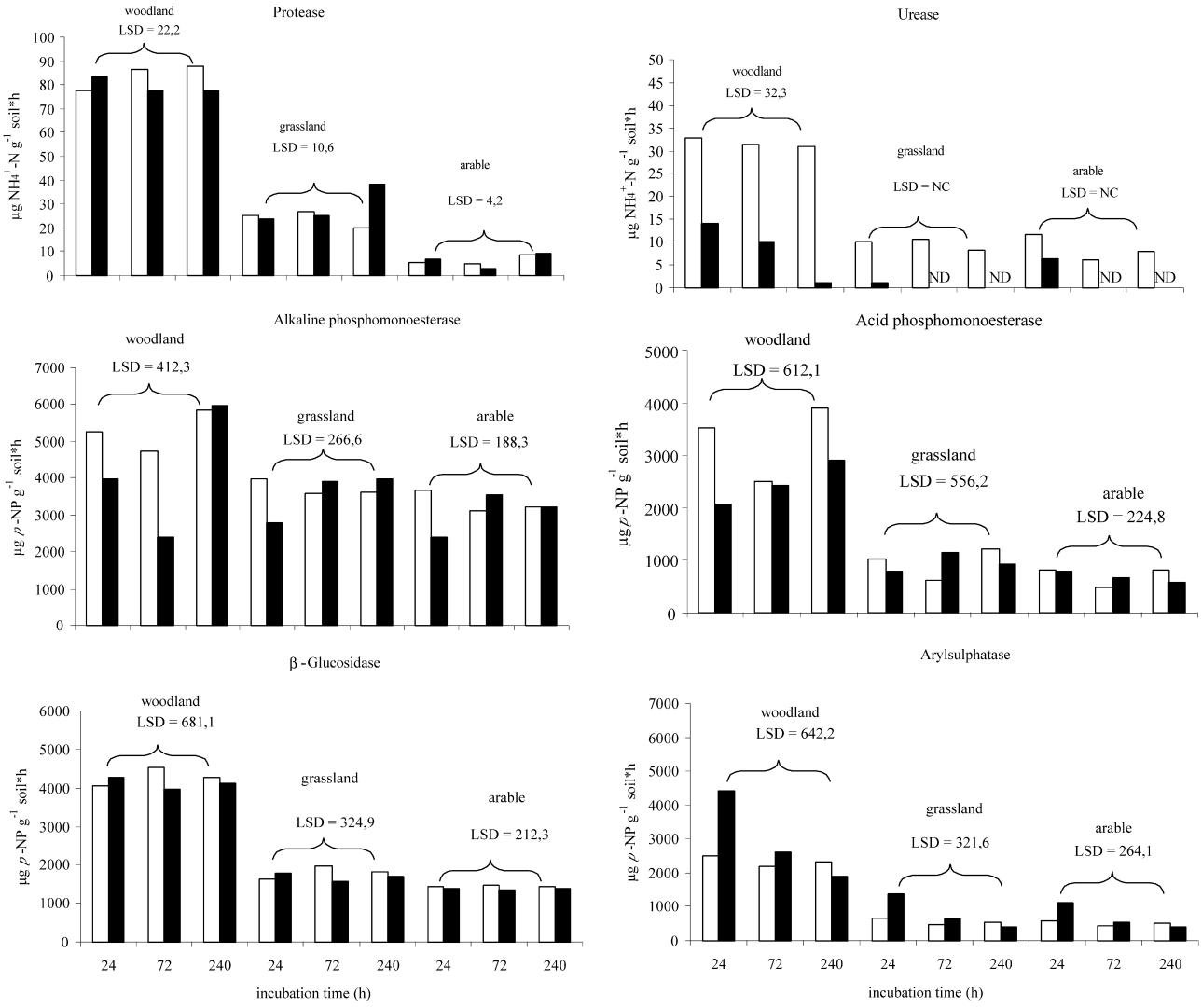

Fig. 4. Hydrolase activity of the soils during the 240 incubation period. □, unfumigated; ■, fumigated; ND, not detectable.

related to the organic matter content of soil (Skujins, 1978; Nannipieri, 1994).

$CHCl_3$ fumigation caused a reduction (mostly significant) in urease, alkaline and acid phosphomonoesterase activities, whereas the decrease of the β-glucosidase activity was not significant (Fig. 3). Protease activity decreased slightly in the woodland and arable soils while it was slightly higher in the grassland soils (Fig. 3). Changes in protease activity were not significant. On the other hand, the arylsulphatase activity was significantly higher at any time of fumigation as compared to values from unfumigated soils (Fig. 3). A possible explanation for the different behaviour of the arylsulphatase activity could be the effect of released reaction products. Arylsulphatase activity is not inhibited by sulphate and sulphite (Al-Khafaji and Tabatabai, 1979) while the other measured hydrolase activities might be inhibited by reaction products.

During the incubation following fumigation phosphomonoesterase and β-glucosidase activities recovered to values not significantly different from those of unfumigated soil incubated for the same period of time (Fig. 4). Protease activity showed only minor changes during the incubation of fumigated and unfumigated soils (Fig. 4). Arylsulphatase activity was reduced in all fumigated and incubated soils reaching values not significantly different from those of the unfumigated soils after 240 h (Fig. 4). The urease activity of fumigated and incubated soils decreased to low levels in the woodland soil and was not measurable in the grassland and arable soils after 72 and 240 h of incubation (Fig. 4). This behaviour could be due to the repression of urease synthesis by *N*-compounds produced by the microbial $NH_4^+ - N$ immobilization during the incubation following the fumigation. Indeed, McCarty et al. (1992) reported that urease repression was not caused by the $NH_4^+ - N$ but by the products synthesised from glutamine, the first organic N compound produced after $NH_4^+ - N$ uptake by microbial cells.

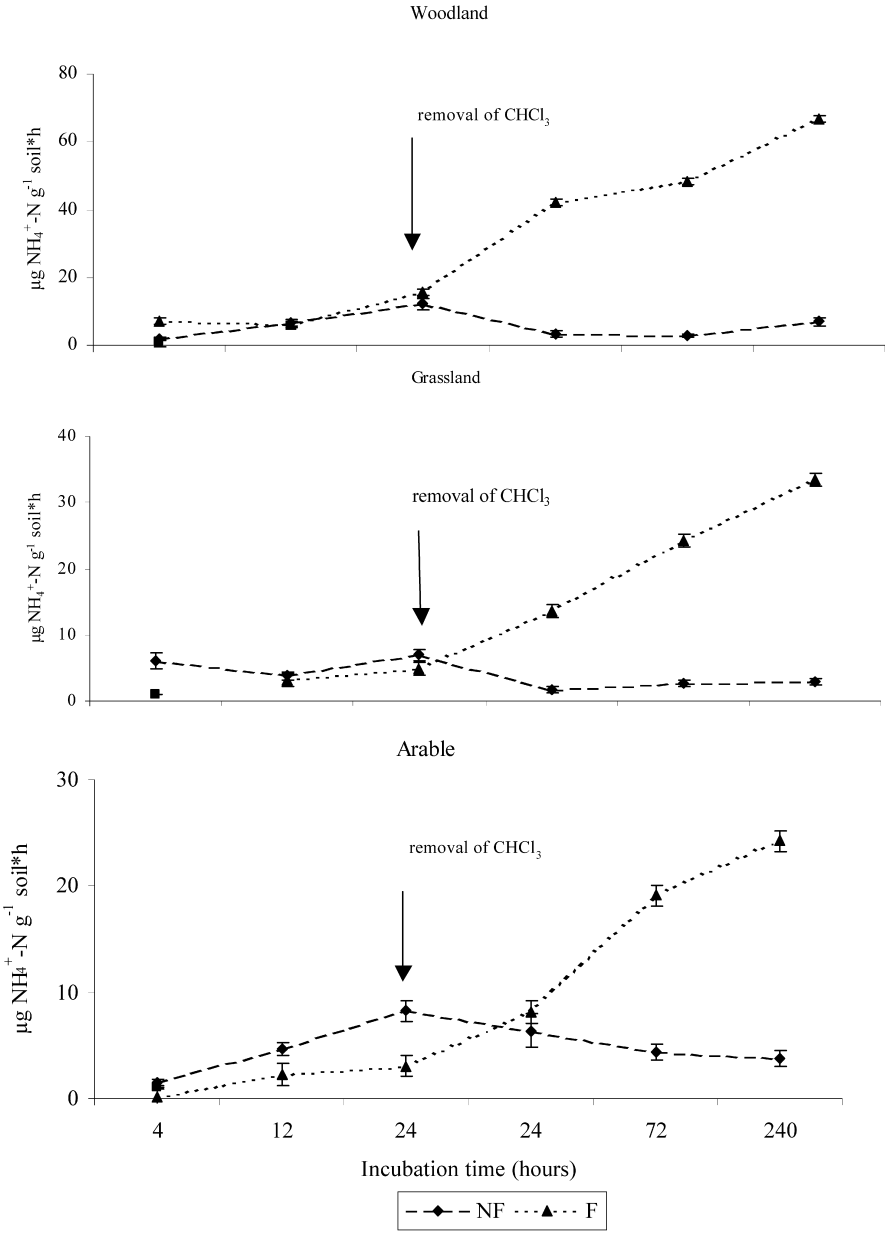

Fig. 5. The exchangeable NH_4^+ − N concentration of soils during and after $CHCl_3$ fumigation. Also reported are the values of the unfumigated soils incubated for the same period of time of the fumigated soils. The error bar is the standard error ($n = 3$) of the mean.

Another possible explanation of the low urease activities in the fumigated and incubated soil might be interference from high NH_4^+ concentration. Bergstrom and Monreal (1998) reported negative urease activities caused by high NH_4^+ content of soil. Our urease assays were based on proper controls, which accounted for the NH_4^+ content of soil, and the NH_4^+ determination by FIAS is sensitive enough to measure changes as small as 0.1 mg of NH_4^+ per l. The extraction of NH_4^+ by KCl or K_2SO_4 before the urease assay markedly alters soil through removal of soluble organic compounds and microbial cells. For this reason ammonium was not extracted before the assays.

Jenkinson and Powlson (1976) reported that the N mineralization was almost five times faster in the fumigated soils than in the unfumigated soils at 10 days of incubation. Indeed, we found that NH_4^+-N concentrations were 4–6 times higher in the fumigated than unfumigated soils after 240 h (Fig. 5).

3.3. Enzyme activities in the presence of the protease inhibitors

The addition of the protease inhibitor cocktail at the highest concentration reduced protease activity up the 50% of initial values in both fumigated and unfumigated

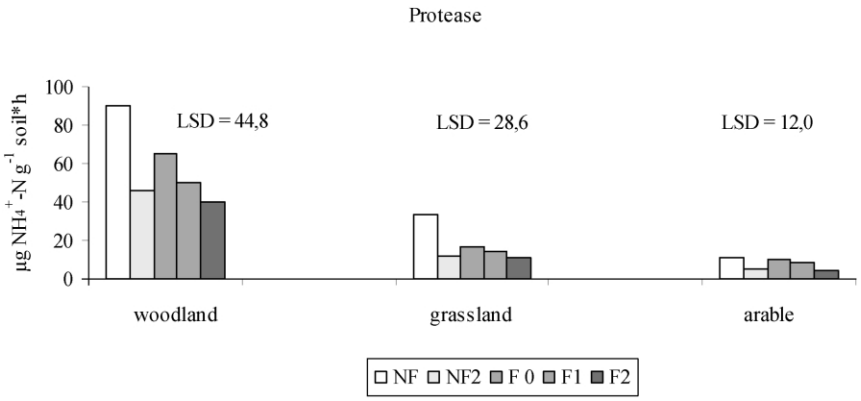

Fig. 6. Protease activity of unfumigated (NF) and soils fumigated (F) for 24 h after the addition of the protease inhibitor solution at two different concentrations: 1 and 2 (the highest and lowest, respectively).

soils (Fig. 6). Protease activity was not completely annulled even at the highest concentration used here. It is reasonable to suppose that some proteases, present as extracellular enzymes adsorbed to clays or embedded in the humic substances, were inaccessible to the inhibitors. Intracellular proteases of living microbial cells located inside soil microaggregates might also not be reached by protease inhibitors. However, it cannot be excluded that even such a broad-mixture of protease inhibitors was not completely effective because some of the soil proteases may require unknown inhibitors. Indeed, these inhibitors were designed for inactivating bacterial proteases and proteases from other microorganisms, such as fungi, could not be affected.

The recovery of enzyme activities following the addition of protease inhibitors shows unequivocally that enzymes released by the lysed cells during $CHCl_3$ fumigation are exposed to the proteolytic attack. The reduction in protease activity led to an increase in the urease and both phosphomonoesterase activities which resulted much higher in the presence than in the absence of the protease inhibitors after the 24 h fumigation period (Fig. 7). The only exception was urease activity of fumigated woodland soil treated with the lowest concentration of inhibitor which remained significantly lower than the control value (Fig. 7). The addition of the protease cocktail inhibitor at two different concentrations did not affect the hydrolase activities of the unfumigated soils (data not shown) with the exception of protease and arylsulphatase activities. The highest concentration of the cocktail inhibitor decreased arylsulphatase activity of the unfumigated soil by 24, 14 and 27% in the woodland, grassland and arable soil, respectively (data not shown). The reduction of arylsulphatase activities of both fumigated and unfumigated soils by the protease inhibitors may depend on the fact that some of the inhibitors also negatively affected arylsulphatase. However, we have no hypotheses on the possible mechanism of inhibition.

4. Conclusions

The approach proposed by Klose and Tabatabai (1999a,b) to determine the intracellular enzyme activity after the $CHCl_3$ fumigation of soils presents the problem that hydrolases released after cell lysis are partially degraded by active proteases. Therefore, a complete inhibition of active proteases is required to determine the intracellular enzyme activities using the fumigation method. There may be other drawbacks. For example, denaturation of released enzymes may occur as a result of differences between intracellular and soil pH. Another serious drawback is the assumption that the enzyme activity of the unfumigated soil represents the extracellular stabilized enzyme activity of soil. The increase in enzyme activities after adding nutrient sources to soils (Nannipieri et al., 2001) or the increase in enzyme activities after the incubation of the fumigated soil probably demonstrate that these increases are associated to microbial growth.

The measurement of the enzyme activities after $CHCl_3$ fumigation may be useful for understanding the mineralisation of microbial debris by the surviving microbial population. The activity of this small microbial population is extraordinarily high during the first 3–4 days of incubation following fumigation. Indeed, the hydrolase activities of the fumigated soils were comparable to those of the unfumigated soils regardless of the differences in biomass content, thus demonstrating their key role in the C, N, P and S mineralisation processes.

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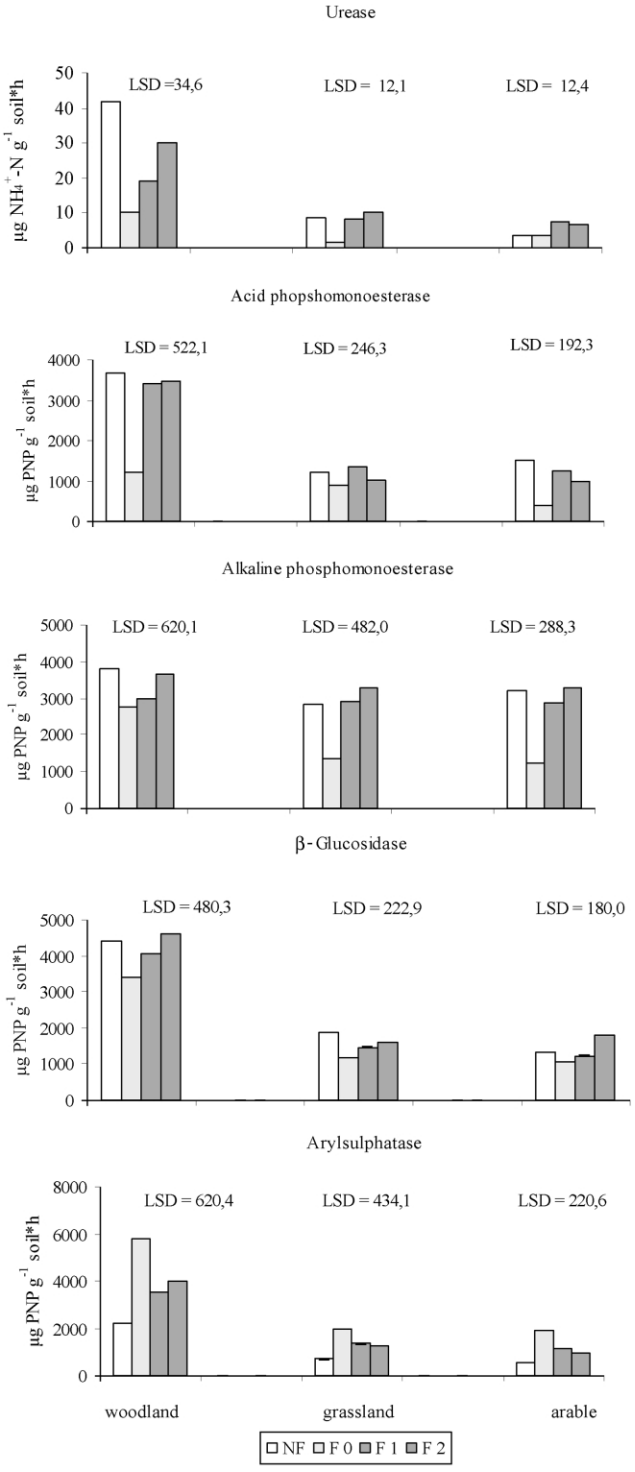

Fig. 7. Hydrolase activities of unfumigated (NF) and fumigated (F) soils after the addition of the protease inhibitor solution at two different concentrations: 1 and 2 (the highest and lowest, respectively).

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