PERGAMON

Soil Biology and Biochemistry 31 (1999) 561–566

Soil Biology & Biochemistry

Determination and depletion kinetics of L-methionine-sulphoximine in soil

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Accepted 23 September 1998

Abstract

L-Methionine-sulphoximine (MSX) is an inhibitor of glutamine synthetase activity which may be used in short-term soil incubation assays for studying its effects on soil N cycling. In order to monitor the fate of MSX in soil a reversed-phase HPLC method equipped with UV-detection has been developed. The fate of the inhibitor was assessed by its recovery after incubation in five contrasting soils. There was a marked decrease of MSX concentration in all cases. The first order kinetic model $f(x) = A\cdot(k_r + k_f\cdot\exp(-x\cdot(k_f + k_r)))/(k_f + k_r)$ was fitted to recovery values and it showed the rate of MSX loss was a time-dependent equilibrium process. The effect of clay minerals in reducing the concentration of MSX in soil solution through adsorption and binding processes was also assessed in two of the five soils after their amendment with mined clay (either kaolinite or montmorillonite). Analytical results showed that the amount of MSX recovered was related negatively to soil CEC values ($r = -0.904$) and total C ($r = -0.931$) and N ($r = -0.952$) content. The weak ability of MSX to block N immobilization in colloid-rich soils could therefore be due to MSX adsorption–desorption by soil colloids which considerably reduce the inhibitor concentration.

1. Introduction

In soil, glutamine synthetase (GS), the enzyme catalysing the reaction between NH_4^+-N and glutamic acid in the biochemical process for inorganic N incorporation into organic compounds (Magasanik and Neidhardt, 1987), is supposed to play a central role in the process of N immobilization (McCarty, 1995).

A method based on the use of the L-methionine-DL-sulphoximine (MSX), an inhibitor of GS activity for easily and accurately measuring gross N mineralization rate was proposed by Nannipieri et al. (1994) and Landi et al. (1995). The premise for this approach was that net N mineralization in MSX-treated soil would equal gross mineralization in the untreated soil. However, during preliminary experiments, this was not the case partly because MSX was only partially effective at blocking the N immobilization process and was itself mineralized thereby contributing to the soil NH_4^+-N pool (Hopkins et al., 1995; Landi et al., 1995; Landi et al., 1999). If MSX is to be used in assays of N mineralization, two questions need to be answered. These are: (i) how efficient is MSX in controlling soil NH_4^+-N immobilization? and (ii) what is the fate of MSX in the soil? It is possible that the inhibitor molecule may be either adsorbed on soil colloids or metabolized by soil microbial community as a nutrient and energy source (Landi et al., 1995; Landi et al., 1999).

Our aim was to follow the short-term (up to 48 h) kinetics of MSX decrease in soil incubation assays, in order to assess the inhibitor concentration effective for controlling the soil NH_4^+-N immobilization process driven by glutamine synthetase. The MSX loss was calculated by the difference between the amount recovered in the soil extract and the amount added. A reversed-phase HPLC method was developed for determining MSX content in soil extracts. Due to its amino acid structure, the inhibitor was determined applying the HPLC methodology for amino acid analysis (Jones et al., 1981; Lookhart and Jones, 1985; White and Hart, 1992). The chromatographic method was based on McAuley's procedure (McAuley, 1995), with modi-

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0038-0717/99/$19.00
PII: S0038-0717(98)00158-8

fications, after pre-column derivatization with *o*-phthalaldehyde (OPA) followed by UV-detection of MSX derivates.

The kinetics of MSX disappearance was investigated in five soils with differing chemical and physical properties. Two of them were subsequently amended with mined clay (either kaolinite or montmorillonite) to manipulate, amongst other properties, the cation exchange capacity (CEC).

2. Materials and methods

2.1. Soils and clay-amended soils

Agricultural and forest soils with differing physical and chemical properties (Table 1) were surface sampled (0 to 20 cm), partially air-dried, sieved (< 2 mm) and stored at 4°C for 15 d before analysis.

Soil pH was measured by a glass membrane electrode in 1:2.5 (w/v) soil:water or soil: 1 M KCl mixtures; total C and total N were determined by a Carlo Erba Na 1500 Autoanalyser (Milan, I); particle size distribution was measured by the pipette method (Day, 1965); the CEC was determined by using a 10% (w/v) $BaCl_2$ H_2O–triethanolamine (2.25%, v/v) solution at pH 8.1 (Società Italiana di Scienza del Suolo, 1985); soil water holding capacity (WHC) was determined according to Forster (1995). Clay characterization in tested soils was performed by X-ray diffraction (XRD) analysis. XRD data (Table 2) were obtained in 2θ intervals from 3° to 15°, using Co-$K_{\alpha 1}$ radiation, with a Philips PW 1710 diffractometer.

The two soils with the smallest clay contents, Passo del Mercante-beech forest soil and Romola soil, were chosen for the preparation of soil–clay mixtures. From the XRD analysis Passo del Mercante-beech forest soil showed results typical for kaolinite and Romola soil for montmorillonite (Table 2). A Twiggs County (Georgia, USA) kaolinite, and a Clay Spur (Wyoming, USA) montmorillonite with cation exchange capacity values of 9.56 and 70.27 meq 100 g^{-1} dry weight, respectively, were from Ward's Natural Science Establishment, Rochester (New York, USA). The two soil–clay mixtures were prepared as follows: mixture 1 (beech forest soil:kaolinite, 65:35, w/w) and mixture 2 (Romola soil:montmorillonite, 75:25, w/w) to give final CEC values of 39.5 and 29.9 meq 100 g^{-1} dry weight, respectively. The mixtures were incubated and processed in the same way as the soils.

2.2. Reagents

MSX was purchased from Sigma (St. Louis, MO). *o*-Phthalaldehyde (OPA) of chromatographic grade was from Pickering Laboratories (Mountain View, CA); boric acid and β-mercaptoethanol (β-MCE) of analytical grade and Whatman No. 42 filter paper were purchased from Carlo Erba Reagenti (Milan, Italy). LiChrosolv gradient grade methanol, tetrahydrofuran (THF) for HPLC, 100% glacial acetic acid and sodium acetate were from Merck (Darmstadt, Germany).

2.3. Soil incubation assay

Moist soils, equivalent to 10 g soil on an oven-dry basis (105°C, 24 h), were amended with 1 μmol g^{-1} dry weight MSX in sufficient water to achieve 50% water holding capacity (WHC) and incubated for 0.5, 1.0, 1.5, 3, 6, 12, 24 and 48 h at 23°C. A zero time control was also prepared for each sample. At the end of the incubation period MSX was recovered by shaking with 50 ml 0.5 M K_2SO_4 (1:5, w/v, soil:solution) for 1 h at room temperature. The soil mixtures were then centrifuged for 15 min at 4000*g*, the supernatant was filtered through a Whatman No. 42 filter paper and the filtrate stored at −20°C.

Table 1
Physical and chemical properties of tested soils

Sampling site	Vegetation cover	Particle distribution (%)			Texture[a]	pH		Total C (%)	Total N (%)	CEC[b]	WHC[c]
		sand	silt	clay		H_2O	KCl				
Bova Marina[d]	uncultivated	11.0	68.0	21.0	Si-L	8.2	7.8	1.40	0.086	12.4	55
Passo del Mercante[d]	beech forest	61.0	32.0	7.0	Sa-L	5.8	4.6	8.11	0.680	45.6	48
Passo del Mercante[d]	pine forest	76.5	15.0	8.5	Sa-L	5.6	4.6	2.47	0.166	17.5	45
Pistoia[e]	horticultural	63.2	17.6	19.2	Sa-L	6.7	6.1	3.89	0.280	15.2	45
Romola[e]	fallow	90.7	3.6	5.7	Sa	7.2	5.9	1.05	0.098	16.9	35

[a] USDA classification. Sa: sand; Sa-L: sandy loam; Si-L: silt loam.[b]meq 100 g^{-1} dry soil.[c]Water holding capacity, ml H_2O 100 g^{-1} dry soil.[d]Reggio Calabria (Southern Italy). Passo del Mercante from 850 m altitude.[e]Firenze (Central Italy).

Table 2
Clay characterization of tested soils

Soil sample	Smectite	Vermiculite	HIV	Mica	Kaolinite	Chlorite	Chlorite/ Vermiculite	Montmorillonite/ Vermiculite
Bova marina	0	+(+)	+ + + +	+ + (+)	+(+)	(+)	0	0
Beech forest[a]	0	0	0	+	+	trace	+	0
Pine forest[a]	0	0	0	+	+	(+)	trace	0
Pistoia	0	+ + + +	+(+)	+ +	+	trace	+(+)	0
Romola	trace	+(+)	+ + (+)	+(+)	+(+)	0	0	+ + (+)

[a] Sampling site: Passo del Mercante (Reggio Calabria).

2.4. MSX determination

2.4.1. Soil extract pretreatment

Before chromatographic analysis, soil extracts were purified by passing 2-ml aliquots through a 0.20-μm pore cellulose-acetate filter (Micro Filtration Systems (MFS), Sierra Court, CA) and then 1 ml aliquots of the filtrate were passed though a C_{18} Sep-Pak™ cartridge (Waters Corporation, Milford, MA). The cartridge was then washed with 1 ml of double-distilled H_2O and the eluates were pooled; purified soil extracts were stored at −20°C before derivatization.

2.4.2. Derivatization procedure

The OPA derivatizing reagent was prepared by mixing: (i) 4.5 ml 0.4 M sodium borate buffer, pH 9.5, previously filtered through a 0.20-μm pore MFS filter, (ii) 0.5 ml of OPA stock solution (50 mg OPA ml^{-1} methanol) and (iii) 80 μl of β-MCE. The derivatizing reagent was freshly prepared every 24 h and kept in the dark in an ice-bath. Purified soil extract (100 μl) was mixed with 200 μl of OPA derivatizing reagent at room temperature in the dark. Exactly 3 min later 100 μl of 5% (v/v) CH_3COOH solution were added and mixed; 20 μl of the resulting solution were injected into the HPLC column.

2.4.3. Chromatographic analysis

Gradient high-performance liquid chromatography (HPLC) was performed on a Perkin-Elmer LC 250 binary pump (Norwalk, CT) equipped with a Rheodyne 7125 injector (20-μl loop) and a 2 cm Supelguard™ guard column and a 15 cm × 4.6 mm i.d. Supelcosil™ C_{18} (5 μm) analytical column from Supelco (Bellefonte, PA). A Perkin-Elmer LC 135 diode array detector operating at 340 nm (band width, 5 nm) and a Perkin-Elmer Omega-2 Analytical Workstation were used for detection and data processing, respectively.

Elution was carried out using a gradient program between two degassed solvents at 1 ml min^{-1} flow rate. Solvent A was 20 mM sodium acetate (pH 5.7):HPLC-grade methanol:THF (83:16:1 by volume); solvent B was HPLC-grade methanol. The mobile phase composition was held at 100% A for the first 10 min; was then brought to 78/22 A/B over the next 10 min (linear gradient), followed by 5 min during which it was brought to 40/60 A/B (linear gradient) and held 5 min before returning the column to the initial conditions (100% A) within a further 10 min. The equilibration time was 10 min and MSX retention time was 17 min under these conditions.

2.5. Data analysis

The data reported are mean values from three replicates and are expressed on an oven-dry soil basis (105°C, 24 h). Simple linear correlation and significant tests were used to investigate the relationships between MSX equilibrium concentration and soil chemical and physical properties. A simple equilibrium equation, forward and reverse rate, first order in both direction, was fitted to analytical data by a non-linear regression software (TableCurve 2D v4.0, Jandel Scientific, San Rafael, CA) using the Levenberg–Marquardt optimization algorithm. Non-linear regressions were repeated at least four times using different initial parameters estimates for each set of data. All runs gave the same parameter estimates. The decay curves were plotted using a SigmaPlot v3.0 (Jandel Scientific) software.

3. Results

Monitoring MSX content in five tested soils under short-term incubation assays (up to 48 h) showed a marked decrease of the inhibitor concentration, with a decreasing pattern for each soil investigated (Fig. 1). The largest and the smallest decreases after 48 h incubation were observed for the Pistoia and Romola soil, respectively. In fact, the initial MSX concentration (1 μmol g^{-1} dry soil) was reduced to 0.38 (in Pistoia soil) and 0.49 μmol g^{-1} dry soil (in Romola soil). MSX recovered in Bova Marina, Beech forest and Pine forest soil, after 48 h incubation, was 0.46, 0.41 and 0.46 μmol MSX g^{-1} dry soil, respectively (Fig. 1).

Recoveries of MSX were used to draw a time-dependent curve showing the inhibitor concentration decay.

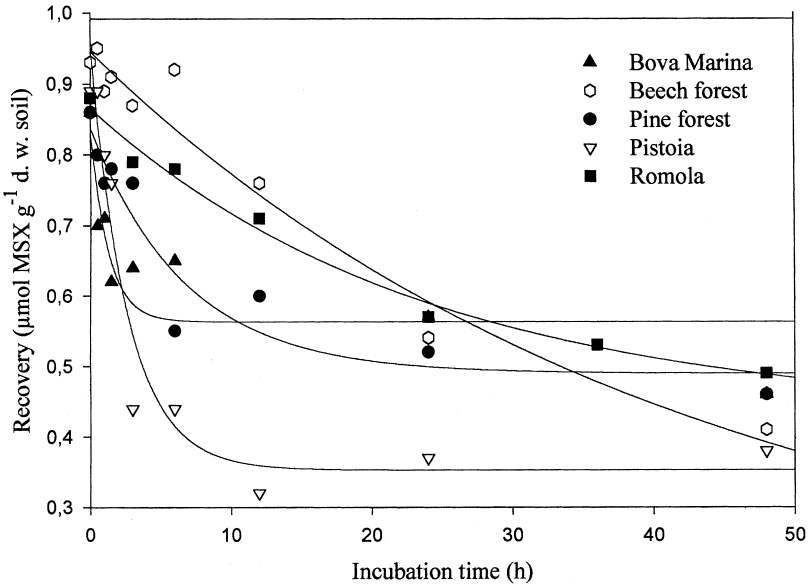

Fig. 1. Kinetics of MSX depletion in five soils under short-term incubation assays.

We found that the rate of MSX loss could be well described by a mathematical model, which is represented by the non-linear, first order equilibrium equation:

$$\frac{dC_t}{dt} = -k_f \cdot C_t + k_r \cdot (C_A - C_t), \quad (1)$$

where C_A is the initial concentration, C_t is the concentration at time t and k_f and k_r are the forward and reverse rate constants, respectively. Eq. (1) may be written in integrated form as the exponential function:

$$f(x) = \frac{A \cdot (k_r + k_f \cdot \exp(-x \cdot (k_f + k_r)))}{k_f + k_r}, \quad (2)$$

where $f(x)$ is the concentration of MSX, in µmol g^{-1} dry soil; x the incubation time in hours; A is the initial concentration of MSX (time zero) and k_f and k_r are the forward and reverse rate constants. Eq. (2) could also be used to describe the MSX disappearance as a

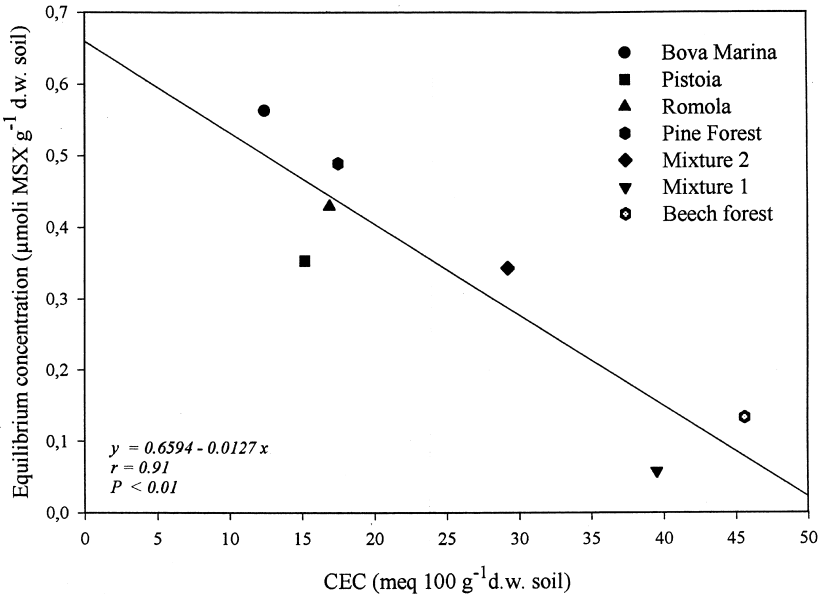

Fig. 2. Linear relationship between CEC and MSX equilibrium concentration values from the five tested soils and the two clay-amended soils.

Table 3
Kinetic parameters of MSX depletion curves

Sample	A [a]	k_{ad}	k_{des}	Equilibrium concentration[b]	r^2
Bova Marina	0.831	0.250	0.524	0.563	0.74
Beech forest[c]	0.945	0.020	0.003	0.133	0.96
Pine forest[c]	0.838	0.062	0.087	0.489	0.91
Pistoia	0.951	0.237	0.140	0.353	0.94
Romola	0.866	0.021	0.021	0.429	0.99
Mixture 1	0.804	0.013	0.001	0.058	0.95
Mixture 2	0.724	0.007	0.007	0.343	0.66

[a] MSX concentration (μmol MSX g^{-1} dry soil) at zero time.[b]Calculated from the relation ($A \cdot k_{des}/(k_{ad}+k_{des})$) and expressed as μmol MSX g^{-1} dry soil.[c]Sampling site: Passo del Mercante (Reggio Calabria).

consequence of an adsorption–desorption equilibrium process occurring at solid–liquid interfaces. If so, k_f and k_r would become the rate kinetic constants for the adsorption (k_{ad}) and desorption (k_{des}) process, respectively. After a period of time the decline reaches an equilibrium concentration (Fig. 1) which may be calculated using the Eq. (2) kinetic parameters found for each tested soil. Equilibrium is attained when the rate of adsorption equals the rate of desorption. Soil kinetic parameters, equilibrium concentrations and coefficients of determination are reported in Table 3. Recovery of MSX at zero time never reached a 100% value: this is probably due to the immediate and irreversible adsorption of the molecule by soil colloids since we suppose that soil microorganisms were not capable of metabolizing a significant amount of MSX in such a short time. At zero time MSX loss ranged between 4.9% (Pistoia soil) to 16.9% (Bova Marina soil).

MSX equilibrium concentrations calculated for each curve (Table 3) were correlated to soil chemical and physical properties. Significant correlations (at $P < 0.01$) were only found with total C ($r = -0.931$), total N ($r = -0.952$) or CEC ($r = -0.904$), suggesting that MSX recovery was strongly influenced by surface interactions between the inhibitor molecule and both mineral and organic soil colloids. No significant correlations were found with other soil properties as pH ($r = 0.573$, in water; $r = 0.607$, in KCl) values and clay ($r = 0.415$), sand ($r = -0.304$) and silt ($r = 0.247$) content.

The MSX decay pattern in the two clay-amended soils was similar to that of the unamended soils (data not shown). The MSX recovered in soil–clay mixture 1 and 2 after 48 h was 0.43 and 0.54 μmol g^{-1} d.w., respectively. As for soils, Eq. (2) well fitted recovery data from mixtures 1 and 2 and the calculated MSX equilibrium concentrations are shown in Table 3.

The linear regression between MSX equilibrium concentrations and CEC values for soils and soil–clay mixtures is reported in Fig. 2.

4. Discussion

The results of MSX recovered after soil incubation (Fig. 1) may help to explain why the MSX concentration (0.5 μmol g^{-1} dry soil), tested by Landi et al. (1995) was ineffective in inhibiting NH_4^+-N immobilization in Pistoia and Romola soils. Probably the rapid decline of MSX concentration may have reduced the inhibitor to an ineffective concentration. An important effect of inhibiting NH_4^+-N immobilization only occurred at the highest concentration (1 μmol MSX g^{-1} dry soil) during the 0–12 h and 0–24 h incubation periods in Pistoia and in Romola soil, respectively. In Pistoia soil, the MSX concentration was reduced to 0.44 and 0.32 μmol g^{-1} dry soil after 3 and 12 h, respectively (Fig. 1). After 12 h the MSX concentration in Romola soil was almost twice as much as that in Pistoia (0.71 versus 0.32 μmol g^{-1} dry soil) and it decreased markedly in Romola soil only after 24 h.

Soil is a complex and heterogeneous environment dominated by a solid phase consisting of particulates of different sizes (Stotzky, 1986). Surface-active particles may play a role in lowering the amount of MSX available to microorganisms through sorption and binding processes. Among soil components clay minerals may adsorb and bind a spectrum of organic molecules (Stotzky, 1986) including amino acids (Greenland et al., 1965a,b; Dashman and Stotzky, 1982). However, in spite of the fact that MSX is an amino acid analogue the rate of MSX loss did not correlate with soil clay content. This is probably because other soil colloids are involved in adsorption and the binding process and clay particles in soil are normally covered by other materials. Needless to say, studies on amino acid adsorption on clays have been carried out with pure homoionic clays (Stotzky, 1986).

The inhibitor, being an amino acid analogue, may be used as a nutrient and energy source by soil microorganisms. Microbial breakdown of MSX has been assumed to occur at low concentrations (Landi et al., 1999) and it has been observed at higher concentrations (Hopkins et al., 1995) in soil. Therefore MSX

mineralization by soil microorganisms cannot be excluded at 1 μmol MSX g^{-1} dry weight. However, the fact that an equilibrium state was reached within a few hours in Pistoia, Bova Marina and Passo del Mercante-Pine forest soils (Fig. 1) and later in Romola in Passo del Mercante-Beech forest soils suggests that adsorption–desorption processes were occurring.

The most significant correlations were found between loss of MSX and the total C and N content suggesting that results were linked to soil organic matter content. The contribution of soil organic fraction to MSX adsorption is presently being investigated.

Moreover, significant correlation was also found with CEC (Fig. 2), which accounts for soil colloids surface activity due to both mineral and organic colloids. It may be possible that the inhibitor, once in soil, is either degraded by microorganisms or irreversibly bound and protected against microbial breakdown by soil colloids; both processes would control the amount of inhibitor available for inhibiting glutamine synthetase activity, i.e. microbial N immobilization. The adsorption and binding of organic substrates to clay minerals usually reduces their availability to microbes (Stotzky, 1986). Furthermore, bound clay-amino acids have been shown to be poorly available to soil microorganisms as a source of C and N, especially to those with a low amount of energy derived from the intracellular metabolism (Dashman and Stotzky, 1986).

The addition of mined clays resulted in a decrease of recovery either in montmorillonite- and in kaolinite-amended soil (Fig. 2). This could be due to either a direct effect involving surface interaction between clay mineral and MSX molecule (e.g. sorption) or an indirect effect on soil microbial ecology; added clay may have modified the physicochemical characteristics of microbial habitats, negatively influencing microbial activity and growth (Stotzky, 1986).

In conclusion, CEC, total C and N contents seem to control the fate of MSX in soil environment. Sorption by soil organic and mineral colloidal surfaces, can markedly influence the amount of inhibitor available to soil microorganisms. This might reduce the efficiency of the MSX in blocking N immobilization. Consequently, the use of MSX in assays of gross N mineralization must be carefully considered especially in colloid-rich soils.

Acknowledgements

The authors are grateful to Dr. M. Sidari and Mr. V. Cianci for technical assistance. The research was funded by the Ministry of University and Scientific and Technological Research (MURST) of Italy and by the European Economic Community.

References

Dashman, T., Stotzky, G., 1982. Adsorption and binding of amino acids on homoionic montmorillonite and kaolinite. Soil Biology & Biochemistry 14, 447–456.

Dashman, T., Stotzky, G., 1986. Microbial utilization of amino acids and a peptide bound on homoionic montmorillonite and kaolinite. Soil Biology & Biochemistry 18, 5–14.

Day, P.R., 1965. Particle fractionation and particle-size analysis. In: Black, C.A. (Ed.), Methods of Soil Analysis, Part 1. American Society of Agronomy, Madison, pp. 545–567.

Forster, J.C., 1995. Determination of water-holding capacity of soil. In: Alef, K., Nannipieri, P. (Eds.), Methods in Applied Soil Microbiology and Biochemistry. Academic Press, London, p. 106.

Greenland, D.J., Laby, R.H., Quirk, J.P., 1965a. Adsorption of amino acids and peptides by montmorillonite and illite. Part 1. Cation exchange and proton tranfer. Transactions of the Faraday Society 61, 2013–2023.

Greenland, D.J., Laby, R.H., Quirk, J.P., 1965b. Adsorption of amino acids and peptides by montmorillonite and illite. Part 2. Physical adsorption. Transactions of the Faraday Society 61, 2024–2035.

Hopkins, D.W., Anderson, L., Scott, S.E., 1995. N and C mineralization in soil amended with the N immobilization inhibitor, methionine sulphoximine. Soil Biology & Biochemistry 27, 377–379.

Jones, B.N., Pääbo, S., Stein, S., 1981. Amino acid analysis and enzymatic sequence determination of peptides by an improved *o*-phthaldialdehyde precolumn labeling procedure. Journal of Liquid Chromatography 4, 565–586.

Landi, L., Badalucco, L., Nannipieri, P., 1995. Changes in inorganic N and CO_2 evolution in soil induced by L-methionine-sulphoximine. Soil Biology & Biochemistry 27, 1345–1351.

Landi, L., Barraclough, D., Badalucco, L., Gelsomino, A., Nannipieri, P., in press. The effect of L-methionine-sulphoximine on N mineralization-immobilization in soil. Soil Biology & Biochemistry, in press.

Lookhart, G.L., Jones, B.L., 1985. High-performance liquid chromatography analysis of amino acids at the picomole level. Cereal Chemistry 62, 97–102.

Magasanik, B., Neidhardt, F.C., 1987. Regulation of carbon and nitrogen utilization. In: Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M., Umbarger, H.E. (Eds.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. American Society of Microbiology, Washington DC, pp. 1318–1325.

McAuley, P.J., 1995. Ammonium metabolism in the green hydra symbiosis. Biological Bulletin 188, 210–218.

McCarty, G.W., 1995. The role of glutamine synthetase in regulation of nitrogen metabolism within the soil microbial community. Plant and Soil 170, 141–147.

Nannipieri, P., Badalucco, L., Landi, L., 1994. Holistic approaches to the study of populations, nutrients pools and fluxes: limits and future research needs. In: Ritz, K., Dighton, J., Giller, K.E. (Eds.), Beyond the Biomass. John Wiley & Sons, Chichester, UK, pp. 231–238.

Società Italiana di Scienza del Suolo, 1985. Metodi Normalizzati di Analisi del Suolo. Edagricole, Bologna.

Stotzky, G., 1986. Influence of soil mineral colloids on metabolic processes, growth, adhesion and ecology of microbes and viruses. In: Huand, P.M., Schnitzer, M. (Eds.), Interactions of Soil Minerals with Natural Organics and Microbes. Soil Science Society of America, Madison, pp. 305–428.

White, J.A., Hart, R.J., 1992. HPLC analysis of amino acids. In: Nollet, L.M.L. (Ed.), Food Analysis by HPLC. Marcel Dekker, New York, pp. 75–115.