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# L-Methionine-sulphoximine affects N mineralisation-immobilisation in soil

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**Abstract**

The isotopic dilution technique was used to determine gross N mineralisation and immobilisation rates of two soils (Pistoia, sand–clay–loam, and Romola, sandy) in the presence of L-methionine-sulphoximine (MSX), an inhibitor of glutamine synthetase (GS) enzyme. We hypothesized that a complete inhibition of this enzyme would block N immobilisation and thus would allow us to determine gross mineralisation rates from net mineralisation rates. Soils were treated with $^{15}N$-labelled ammonium sulphate at 10.1 atom% (10 μg N $g^{-1}$ soil) plus unlabelled potassium nitrate (10 μg N $g^{-1}$ soil), with or without MSX (1 μmol $g^{-1}$ soil) and incubated at 25°C; selected variables were determined at 0, 6, 24, and 48 h of incubation. The fate of MSX was followed by its recovery from the two soils by extraction, derivatization and HPLC analysis. The addition of MSX to both soils increased gross and net mineralisation; the immobilisation data suggested that MSX had, to some extent, inhibited N immobilisation, although its effectiveness appeared to decrease with time in the Pistoia soil. This behaviour was consistent with the greater rate of MSX disappearance in this soil, probably due to adsorption on soil colloids (the Pistoia soil had 19% clay compared to 6% for Romola). The correspondence between the N in the unrecovered MSX and the additional cumulative gross N mineralisation following MSX addition in Romola soil strongly suggested that MSX was also microbially degraded. Inhibition of N immobilisation by MSX was inferred from: (1) The ratio $^{15}N$ excess in the microbial biomass to the mean $NH_4^+$ abundance throughout; (2) the mass balance of the inorganic N pool. The second method of calculation suggested that, with the inhibition of $NH_4^+$ immobilisation by MSX, soil microorganisms switched to immobilising $NO_3^-$. The premise for using MSX was that, in its presence, net mineralisation would equal gross mineralisation in the untreated soil. This was not the case because MSX was only partially effective at blocking immobilisation, MSX was itself mineralised releasing N, and the soil microorganisms appeared to switch to $NO_3$-N as an N source. 

## 1. Introduction

The complexity of the soil nitrogen cycle arises from the number of processes transforming N and from the fact that many are simultaneous. Thus, ammonium is produced by the mineralisation of soil organic matter and simultaneously consumed by nitrification, plant uptake, immobilisation (assimilation by soil microorganisms) and loss. In recent years, $^{15}N$ isotope dilution techniques have been used increasingly to determine the gross rate of $NH_4^+$ production (mineralisation) unconfounded by the processes that consume it (Kirkham and Bartholomew, 1954; Barraclough et al., 1985; Nishio et al., 1985). Because the methodology for isotope dilution is complicated and requires expensive instrumentation, there is a clear need for a simpler way of determining gross rates of mineralisation in soil.

One approach would be to block N immobilisation thereby allowing mineralisation to be determined from the change in mineral-N over time (providing N losses are insignificant). Nannipieri et al. (1994) have suggested the use of L-methionine-sulphoximine

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(MSX) which inhibits the glutamine synthetase (GS) enzyme, part of the GS–GOGAT system responsible for the microbial assimilation of $NH_4^+$ at concentrations $<0.1$ mM. However this approach relies on MSX only affecting immobilisation. If MSX were itself to be mineralised by soil microorganisms, the resultant release of N (MSX is $C_5H_{12}N_2O_3S$) would invalidate estimates of gross N mineralisation. Similarly, if inhibiting assimilation increases the catabolism of cytoplasmic protein-N, the approach could give anomalously high estimates of gross mineralisation (Reitzer and Magasanik, 1987). Earlier work has suggested that MSX is effective at inhibiting N immobilisation in soil (Landi et al., 1995). However, there are indications that C mineralisation is increased following the inhibitor addition to soil, raising the possibility of MSX mineralisation (Hopkins et al., 1995; Landi et al., 1995). For MSX to be useful, it must be demonstrable that net mineralisation in its presence is equal to gross mineralisation in its absence. We used $^{15}N$ isotope dilution and enrichment to determine: (1) Whether MSX inhibits $NH_4$-N immobilisation; (2) if gross N mineralisation is increased following its addition to soil; and (3) whether net mineralisation in the presence of MSX equals gross mineralisation in its absence.

## 2. Materials and methods

### 2.1. Soils and experimental design

Two soils were used: a sandy clay loam (Pistoia) and a sandy soil (Romola). Samples were collected in spring from the 0–20 cm horizon, sieved moist ($<2$ mm) and stored at 4°C for 1 month. Selected properties of the Pistoia and Romola soil are, respectively: sand 63 and 90%; silt 18 and 3%; clay 19 and 6%; organic C 3.64 and 0.86%; total N 0.26 and 0.08%; pH ($H_2O$) 6.7 and 7.2 (Landi et al., 1995). Seven days before the experiment, soils were conditioned at room temperature to stabilize the flush of mineralisation after which 20 g fresh soil samples were placed in 500 ml polyethylene bottles and conditioned at 25°C for 24 h with dissolved glucose (300 μg glucose-C $g^{-1}$ dry soil) to reduce the ammonium concentration and increase the soil microbial population. Thereafter 10 μg N $g^{-1}$ dry soil as $(^{15}NH_4)_2SO_4$ (10.1 at%) and 10 μg N $g^{-1}$ dry soil as unlabelled $KNO_3$ with or without 1 μmole $g^{-1}$ dry soil MSX (Sigma Chemical Company) were added in sufficient water to achieve 50% water holding capacity (WHC). The soils were immediately shaken for 10 s on a vortex mixer to ensure uniform distribution and the flasks stoppered and incubated for 0, 6, 24 and 48 h at 25°C. Each treatment was replicated four times.

### 2.2. Analyses

Inorganic N was extracted by shaking 20 g fresh soil for 1 h with 100 ml 1 M KCl containing 5 mg $l^{-1}$ phenylmercuric acetate, an inhibitor of enzyme activity (Keeney and Nelson, 1982). Soil suspensions were filtered through glass fibre filter (Whatman GF/A) and the extracts stored at 4°C prior to analyses for $NH_4$-N and $NO_3$-N by a flow injection analyser. A gas diffusion-mixed indicator method was used for $NH_4$-N; $NO_3$-N was determined after reduction to $NO_2^-$ with copperized cadmium and reaction with sulfanilamide-napthylethylenediamine (Keeney and Nelson, 1982).

For $^{15}N$ analysis, soil extracts (about 80 ml) were placed in 250 ml Erlenmeyer flasks with 0.5 g MgO. The liberated $NH_3$ was trapped on 5 mm glass fibre discs (Whatman GF/D) acidified with 10 μl of 2.5 M $KHSO_4$ placed on a stainless steel hook fixed to the rubber bung stoppering the flask (Brooks et al., 1989). The flasks were diffused for 5 d after which the discs were removed, dried over anhydrous $CaSO_4$ and analysed for $^{15}N/^{14}N$ isotope ratio by mass spectrometer (VG Micromass 622). The solution remaining in the flasks was shaken unstoppered overnight to remove any traces of $NH_4$-N and then 0.2 g Devarda's alloy added with a new acidified glass fibre disc to recover the $NO_3$-N for isotopic analysis as before.

Microbial biomass N was determined by a modification of the fumigation–extraction method (Brookes et al., 1985). After the first KCl extraction the soil remaining on the glass fibre filter was washed and refiltered twice under suction with 100 ml of 1 M KCl to remove any residual labelled or unlabelled inorganic N. At each filtration a new glass fibre filter was used and the previous KCl washing discarded. The washed soil samples were fumigated under $CHCl_3$ in a desiccator for 24 h and then extracted in 100 ml 0.5 M $K_2SO_4$ for 1 h (Brookes et al., 1985). An aliquot of this extract (50 ml) was treated with 10 ml concentrated $H_2SO_4$ (96%) and 1 ml of 0.29 M CuSO and then, after driving off excess water by heating at 120°C for 2 followed by 1 h at 180°C, it was digested for 3 h at 360°C. After collection in 1 ml 0.0025 M $H_2SO_4$, total N in the digests was determined by steam distillation and back titration with 0.005 M NaOH. The $^{15}N$ enrichment of the microbial biomass was determined as above.

### 2.3. Calculation of gross mineralisation and immobilisation

Gross N mineralisation rates were calculated from the isotopic dilution equation (Kirkham and Bartholomew, 1954; Powlson and Barraclough, 1993):

$$*A_t/*A_0 = (A_0/A_t)^{m/\theta}$$

where $m$ is the gross rate of mineralisation, $\theta$ is the rate at which the $NH_4$-N pool changes size $[(A_t - A_0)/t)]$, $*A_0$ and $*A_t$ are the $^{15}N$ excess atom% of the $NH_4$-N pool at time zero and $t$, respectively, and $A_0$ and $A_t$ are the size of the ammonium pool at time zero and $t$, respectively.

Cumulative immobilisation from the $NH_4$-N pool was calculated from the equation (Powlson and Barraclough, 1993):

$$I = B_N \frac{*B}{[*A]}$$

where $I$ is cumulative immobilisation from the $NH_4$-N pool, $B_N$ is the biomass N (determined from the N flush following fumigation multiplied by 2.2), $*B$ is the $^{15}N$ atom% excess in the biomass and $[*A]$ is the mean atom% excess in the $NH_4$-N pool over the experiment. This equation yields the N in the biomass at time $t$ derived from the $NH_4$-N pool and is not strictly a gross measurement; thus any N immobilised and subsequently exported by the biomass in metabolites or through death will be excluded.

### 2.4. Fate of MSX

In a parallel experiment, the fate of MSX was determined from the recovery of MSX from similar amount (10 g) of the two soils after addition of 1 μmol of MSX $g^{-1}$. The soils were at 50% WHC and incubated at 23°C for 0, 3, 6, 12, 24 or 48 h. Glucose was not added in this experiment to ensure that MSX was the main C source. Soils were extracted with 50 ml 0.5 M $K_2SO_4$ (soil:solution ratio 1:5) for 1 h, centrifuged for 15 min at 4000 g, filtered through Whatman 42 filter paper and stored at −20°C prior to analysis.

Aliquots of the extracts (2 ml) were cleaned by passing through 0.2 μm pore cellulose–acetate filter (MicroFiltration Systems, Sierra Court, CA) and then 1 ml was passed through a $C_{18}$ Sep-Pak™ cartridge (Waters Corporation, Milford MA). The cartridge was washed with 1 ml of purified water and eluates were combined; the filtered extracts were stored at −20°C before derivatization with *o*-phthalaldehyde (Pickering Laboratories, Mountain View, CA) and separation on a 15 cm × 4.6 mm i.d. Supelcosil™ $C_{18}$ (5 μm) analytical column from Supelco using methanol: sodium acetate stepped gradient (Jones et al., 1981; McAuley, 1995). HPLC analysis was carried out on a Perkin-Elmer LC 250 binary pump equipped with a Perkin-Elmer LC 135 diode array detector operating at 340 nm (band width 5 nm) for UV detection of MSX derivates.

### 2.5. Statistical treatment of results

Data reported are mean values from four replicates with relative standard deviations (SD). Moreover, treatments were compared for two factors (presence or not of MSX, and time of incubation) by two-way analysis of variance (ANOVA). Least significant difference (LSD) was calculated for the 95% confidence level and is indicated in the graphs.

## 3. Results

The addition of MSX had a marked effect on soil $NH_4$-N concentrations in both soils but the effects on $NO_3$-N and biomass N were smaller (Fig. 1). In the absence of MSX, soil amounts of $NH_4$-N declined with time; MSX addition resulted in an increase in both soils. In the Romola soil the drop in $NH_4$-N in the absence of MSX did not appear to be matched by an increase in $NO_3$-N or in biomass-N. In the Pistoia soil, the increase in $NO_3$-N was greater than the decrease in $NH_4$-N but the biomass $N$ results were variable with time and showed no clear trend. These results illustrate the difficulty in interpreting soil inorganic and biomass $N$ data without information about the rates of the underlying processes.

Fig. 2 shows the $^{15}N$ data for the two soils. In both, the presence of MSX resulted in a more rapid decline in $^{15}NH_4^+$. The $^{15}N/^{14}N$ ratio in the $NO_3$-N showed contradictory trends; in Romola, MSX resulted in an increase in $^{15}N$ compared to control, while in Pistoia it was the reverse. The biomass-$^{15}N$ results were more clear cut. In both soils, MSX reduced the $^{15}N$ of the biomass compared to control. In Romola, in the presence of MSX, biomass $^{15}N$ remained constant over 48 h; in the absence of MSX it almost doubled. In Pistoia, in the presence of MSX, biomass $^{15}N$ again remained constant over 48 h; in the absence of MSX, biomass $^{15}N$ increased significantly.

Gross mineralisation and immobilisation rates, calculated by the methods of Powlson and Barraclough (1993) are shown in Table 1. For both soils, at all time intervals, the presence of MSX significantly increased gross N mineralisation compared to control. The gross mineralisation results for the Romola control soil for the period 0–48 h were very low. The $NH_4$-N immobilisation rates, determined from the biomass N and $^{15}N$ results and the mean $^{15}N$ abundance of the ammonium pool, indicated that MSX did reduce $NH_4$-N immobilisation compared to control.

The recovery of MSX from the two soils, determined in the parallel experiment, declined over time but at every sampling, more was recovered from Romola than Pistoia (Table 2).

## 4. Discussion

The addition of MSX to both soils increased gross and net mineralisation and the immobilisation data

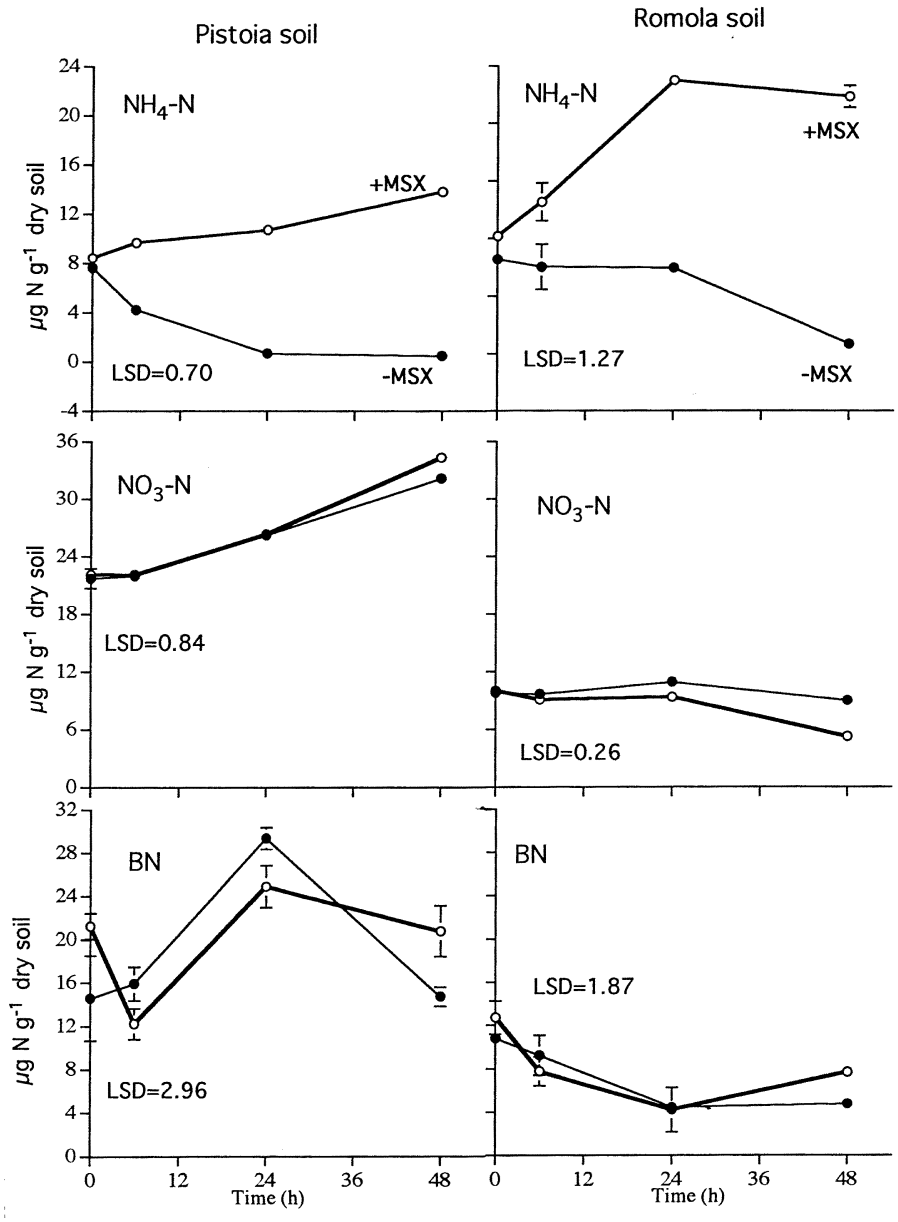


Fig. 1. Ammonium ($NH_4$-N), nitrate ($NO_3$-N) and microbial biomass N (*B*N) contents throughout the incubation in control (–MSX) and MSX-treated (+MSX) soils. Bars indicate standard deviations. Lead significant difference (LSD) for each variable refers to comparisons for two factors (presence or not of MSX, and time of incubation), calculated at the 95% confidence level.

suggested that MSX had, to some extent, inhibited N immobilisation, although its effectiveness appeared to decrease with time in the Pistoia soil. This behaviour was consistent with the greater rate of MSX disappearance in the Pistoia soil, either due to adsorption on soil colloids (the Pistoia soil had 19% clay compared to 6% for Pistoia) or more rapid microbial degradation of MSX.

The disappearance of MSX could be due to microbial degradation or irreversible adsorption on clay colloids or organic matter. In the Romola soil the additional gross N mineralisation over 24 h in the presence of MSX was 376 ng N $g^{-1}$ $h^{-1}$ (645–269). Over 24 h this resulted in an extra 9.02 µg N $g^{-1}$ mineralised. The addition of N as MSX was 28 µg $g^{-1}$. After 24 h, 43% of the MSX was unaccounted for equivalent to 12.04 µg N $g^{-1}$. The correspondence between the N in the unrecovered MSX and the additional cumulative gross N mineralisation following MSX addition strongly suggested that MSX was microbially degraded in this soil and that the increase in gross N mineralisation was due to its decomposition rather than an increase in the catabolism of cytoplasmic protein-N resulting from the inhibition of N assimilation (Reitzer and Magasanik, 1987).

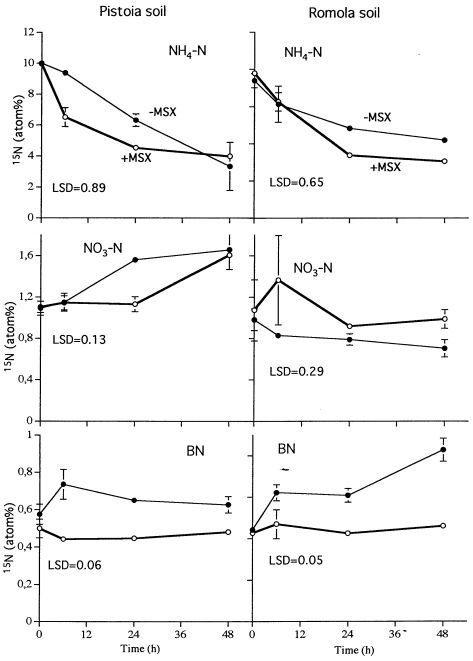


Fig. 2. Labelled ammonium ($NH_4$-N), nitrate ($NO_3$-N) and microbial biomass ($B$N) $^{15}$N (atom%) contents throughout the incubation in control (−MSX) and MSX-treated (+MSX) soils. Bars indicate standard deviations. Least significant difference (LSD) for each variable refers to comparisons for two factors (presence or not of MSX, and time of incubation), calculated at the 95% confidence level.

In Pistoia soil the additional gross N mineralisation over 24 h in the presence of MSX was 6.41 μg N g$^{-1}$. The recovery of MSX at 24 h in Pistoia was 37% implying 63% or 17.6 μg N g$^{-1}$ either degraded or adsorbed. Thus in this soil the increase in gross N mineralisation was considerably less than the shortfall in MSX recovery suggesting that some irreversible adsorption on clay colloids was occurring.

These considerations suggested that MSX was probably mineralised in the Romola soil and possibly in the Pistoia. Despite this, the biomass $^{15}$N data suggested that MSX was effective in inhibiting N assimilation. The biomass N results were, however, less clear. The immobilisation results in Table 1 were calculated from the mean pool $^{15}$N abundance of the $NH_4^+$ pool, the biomass $^{15}$N and the biomass N determined from the N flush following fumigation. There was some evidence that the $k_N$ of recently immobilised N was lower than the average of 0.45 often used (Bremer and van Kessel, 1990). In view of this possibility, N immobilisation was recalculated in a way that it did not use the biomass N content, only its $^{15}$N abundance. The calculation assumed: (1) That while there might be problems with $k_N$, the biomass $^{15}$N determined on the N flush was representative of the whole biomass. There was some evidence from the work of Brookes et al. (1985) that while $B_N$ was influenced by the length of fumigation, the $^{15}$N of the flush was not; (2) $B_N$ was the same in the presence or absence of MSX at $t = 0$; this would imply that the high values in the Pistoia soil were due to the inaccuracy of the fumigation–extraction method when used shortly after glucose addition; and (3) that no loss of N occurred from the biomass.

With these assumptions we obtained the following mass balance for biomass $^{15}$N at $t = 0$.

$$^{15}B\mathrm{N}_0 = B\mathrm{N}_0 \cdot {}^{15}B_0$$

where $B\mathrm{N}_0$ is the biomass N at $t = 0$, $^{15}B_0$ is the $^{15}$N abundance of the biomass N at $t = 0$ (taken as 0.37 at%) and $^{15}B\mathrm{N}_0$ is the amount of $^{15}$N in the biomass at $t = 0$.

At time $t$, following assimilation of labelled $NH_4^+$, the amount of $^{15}$N in the biomass is given by:

$$^{15}B\mathrm{N}_t = {}^{15}B\mathrm{N}_0 + I \cdot [{}^{15}A]$$

where $I$ is the amount of N immobilised and $[^{15}A]$ is the mean atom% of the $NH_4^+$ pool over the time.

Table 1. Effect of MSX on gross N mineralisation and immobilisation rates as determined by isotope dilution (Powlson and Barraclough, 1993)

| Interval time (h) | Gross N mineralisation rate (ng N g$^{-1}$ h$^{-1}$) | | N immobilisation rate (ng N g$^{-1}$ h$^{-1}$) | |
|---|---|---|---|---|
| | control | + MSX | control | + MSX |
| *Pistoia soil* | | | | |
| 0–6 | 66 ± 16[a] | 735 ± 195 | 44 | 16 |
| 0–24 | 62 ± 8 | 329 ± 25 | 9 | 5 |
| 0–48 | 61 ± 6 | 221 ± 6 | 7 | 4 |
| *Romola soil* | | | | |
| 0–6 | 412 ± 271 | 548 ± 200 | 33 | 13 |
| 0–24 | 269 ± 29 | 645 ± 28 | 9 | 3 |
| 0–48 | 71 ± 8 | 354 ± 21 | 8 | 2 |

[a] Standard deviation.

Table 2. Recovery of L-methionine-sulphoximine

| Time (h) | Pistoia soil (% of the added amount) | Romola soil (% of the added amount) |
|---|---|---|
| 0 | 89 ± 1[a] | 88 ± 1 |
| 3 | 44 ± 4 | 79 ± 3 |
| 6 | 45 ± 5 | 77 ± 6 |
| 12 | 32 ± 2 | 71 ± 4 |
| 24 | 37 ± 2 | 57 ± 5 |
| 36 | nd | 53 ± 3 |
| 48 | 38 ± 1 | 49 ± 2 |

The $^{15}N$ atom% of the biomass at time $t$ is then given by:

$$^{15}B_t = (0.0037 \cdot BN_0 + I \cdot [^{15}A])/(B\mathrm{N}_0 + I)$$

which after rearranging and recasting in terms of $^{15}N$ excesses gives:

$$I/B\mathrm{N}_0 = 1/([^{15}A^*]/^{15}B_t^* - 1)$$

where superscript * indicates $^{15}N$ atom% excess (i.e. above natural abundance).

This equation yields immobilisation, $I$, as a multiple of the initial biomass size and uses only the $^{15}N$ data, not the $B_N$ results. Table 3 gives the results calculated in this way. They confirmed those in Table 1 and showed that MSX inhibited $NH_4$-N immobilisation in these two soils, though not completely, and that in the Pistoia soil the efficacy decreased over time.

It should be noted that all the immobilisation calculations so far have concentrated on $NH_4^+$ immobilisation. The biomass $^{15}N$ data indicated that $NH_4^+$ immobilisation was reduced by MSX. However, when full mass balance for the experiments was prepared using the measured gross mineralisation data, immobilisation, estimated by difference, was unaffected by the presence of MSX. The Romola soil over 24 h is used as an example in Table 4.

Table 5 gives all the results calculated in this way, although those for the Romola control soil for the 0–48 h period might be unreliable for the reasons noted above. The immobilisation rates determined in this way were greater than those for $NH_4$-N calculated from the biomass $^{15}N$ data (Table 1) and the difference between the MSX and control treatments much smaller than the same differences of Table 1. This apparent contradiction might be explicable in terms of $NO_3$-N immobilisation (Rice and Tiedje, 1989). If, following the blocking of $NH_4$-N immobilisation, microorganisms switched to $NO_3$-N, overall N immobilisation could remain largely unchanged even though $NH_4$-N immobilisation was partially blocked.

Table 3. N immobilisation calculated using the ratio of $^{15}N$ atom excess in the biomass to the mean $NH_4^+$ pool abundance. Values are reported as percentage of the initial biomass N

| Interval (h) | Pistoia soil | | Romola soil | |
|---|---|---|---|---|
| | control | + MSX | control | + MSX |
| 0–6 | 4.1 | 0.9 | 4.6 | 1.7 |
| 0–24 | 3.6 | 1.3 | 5.1 | 1.3 |
| 0–48 | 4.1 | 2.3 | 9.2 | 2.3 |

Table 4.

| | − MSX | + MSX |
|---|---|---|
| Mineral N $t = 0$ | 20.3 | 22.2 |
| Gross mineralisation | 6.5 | 15.5 |
| Calculated N $t = 24$ | 26.8 | 37.7 |
| Measured | 20.8 | 32.3 |
| Estimate of immobilisation | 6.0 | 5.4 |

Overall these results indicate that MSX was partially effective as an inhibitor of $NH_4$-N immobilisation in soil but that its efficacy was reduced with time probably due to microbial degradation and adsorption on clay colloids. The increase in gross N mineralisation following MSX incorporation was consistent with its microbial degradation and this, with the possibility that microorganisms switched to $NO_3$-N as an N source, would confound its use in determining gross N mineralisation from net mineralisation measurements. For MSX to be useful in determining gross mineralisation from net mineralisation measurements it must be shown that net mineralisation in the presence of MSX is equal to gross mineralisation in its absence. Given the evidence presented above, it is not surprising that in neither of these two soils was that the case. Thus, for the Pistoia soil estimates of gross N mineralisation using net mineralisation in the presence of MSX overestimated gross mineralisation about 4-fold, largely because of the release of N from MSX mineralisation. The conclusion in the Romola soil was similar. Over 24 h, isotope dilution gave a gross N mineralisation in the absence of MSX of 269 ng N $g^{-1}$ $h^{-1}$; net mineral-

Table 5. Gross N immobilisation rates calculated by the inorganic N mass balance method

| Interval (h) | Pistoia soil (ng N $g^{-1}$ $h^{-1}$) | | Romola soil (ng N $g^{-1}$ $h^{-1}$) | |
|---|---|---|---|---|
| | control | + MSX | control | + MSX |
| 0–6 | 600 | 520 | 520 | 310 |
| 0–24 | 160 | 60 | 240 | 220 |
| 0–48 | | ? | 210 | 250 |

? indicates the calculated immobilisation was negative.

isation in the presence of MSX was 425 ng N $g^{-1}$ $h^{-1}$ (645–22).

In conclusion it would seem impossible to set up a method based on MSX in soil to determine gross N mineralisation from net mineralisation measurements. The premise for using MSX was that, in its presence, net mineralisation would equal gross mineralisation in the untreated soil. This was not the case, partly because MSX was only partially effective at blocking immobilisation, partly because MSX was itself mineralised releasing N and partly because the soil microorganisms appeared to switch to $NO_3$-N as an N source.

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