Biol Fertil Soils (1989) 7:180–185

Biology and Fertility of Soils

Synthesis of ^{15}N-labelled microbial biomass in soil in situ and extraction of biomass N

F. Azam*, R. L. Mulvaney, and F. J. Stevenson

Department of Agronomy, University of Illinois, Urbana-Champaign, IL 61801, USA

Summary. A Pakistani soil (Hafizabad silt loam) was incubated at 30 °C with varying levels of ^{15}N-labelled ammonium sulphate and glucose (C/N ratio of 30 at each addition rate) in order to generate different in-situ levels of ^{15}N-labelled microbial biomass. At a stage when all of the applied ^{15}N was in organic forms, as biomass and products, the soil samples were analysed for biomass N by the chloroform ($CHCl_3$) fumigation-extraction method, which involves exposure of the soil to $CHCl_3$ vapour for 24 h followed by extraction with 500 m*M* K_2SO_4. A correction is made for inorganic and organic N in 500 m*M* K_2SO_4 extracts of the unfumigated soil. Results obtained using this approach were compared with the amounts of immobilized ^{15}N extracted by 500 m*M* K_2SO_4 containing different amounts of $CHCl_3$. The extraction time varied from 0.5 to 4 h.

The amount of N extracted ranged from 27 to 270 μg g^{-1}, the minimum occurring at the lowest (67 μg g^{-1}) and the maximum at the highest (333 μg g^{-1}) N-addition rate. Extractability of biomass ^{15}N ranged from 25% at the lowest N-addition rate to 65% for the highest rate and increased consistently with an increase in the amount of ^{15}N and glucose added. The amounts of both soil N and immobilized ^{15}N extracted with 500 m*M* K_2SO_4 containing $CHCl_3$ increased with an increase in extraction time and in concentration of $CHCl_3$. The chloroform fumigation-extraction method gives low estimates for biomass N because some of the organic N in K_2SO_4 extracts of unfumigated soil is derived from biomass.

Key words: Extractability ratios – Microbial biomass – N immobilization-remineralization – Priming effect

* *Permanent address and address for offprint requests:* Soil Biology Division, Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan

The chloroform fumigation-incubation method proposed by Jenkinson and Powlson (1976) has been frequently used for the estimation of biomass N using the expression, $B_N = F_N/k_N$, where B_N is the biomass N, F_N is the difference between the amount of N mineralized in fumigated and unfumigated soil under a particular set of incubation conditions and k_N is a constant representing the proportion of biomass N mineralized during a fixed incubation period. The value of k_N, which has been experimentally determined by Jenkinson (1976), Adams and Laughlin (1981), and Voroney and Paul (1984) is highly variable (Voroney and Paul 1984). In addition, the N mineralized in the fumigated soil during the incubation may be underestimated due to net immobilization by the surviving microbial population or denitrification (Voroney and Paul 1984; Brookes et al. 1985a; Azam et al. 1986).

Recently, Brookes et al. (1985b) proposed a new method that requires exposure of the soil to $CHCl_3$ vapour for 24 h, followed by extraction with 500 m*M* K_2SO_4 and determination of inorganic plus organic N in the extract. With this $CHCl_3$ fumigation-extraction method, biomass N is calculated as the difference between the amount of N extracted immediately after fumigation and the amount extracted from the unfumigated soil at the time the fumigation began. According to Brookes et al. (1985b), the chloroform fumigation-extraction method has several advantages over the conventional chloroform fumigation-incubation method (Jenkinson and Powlson 1976). Both methods are believed to measure the same pool of soil N because (1) the amount of N extracted from soil after 5 days of fumigation was virtually identical to the amount of N mineralized from the biomass by incubation of the fumigated soil, and (2) similar amounts of labelled N were obtained when the two methods were compared using grassland soils previously treated with ^{15}N labelled fertilizer (Brookes et

al. 1985b). However, these findings do not necessarily indicate the exact proportion of biomass N recovered by the chloroform fumigation-extraction method, because the biomass content of the soils used by Brookes et al. (1985b) was not known. In our work, therefore, known quantities of microbial biomass were established in soil in situ, and a comparison was made of the extractability of the biomass N by the chloroform fumigation-extraction method and by direct extraction of soil with 500 m*M* K_2SO_4 containing different amounts of $CHCl_3$ (chloroform extraction method). Our aim was to study the effectiveness of $CHCl_3$ contained in 500 m*M* K_2SO_4 for extraction of biomass N, as compared to biomass N estimated by the chloroform fumigation-extraction method of Brookes et al. (1985b).

Materials and methods

The Hafizabad silt loam soil was collected from the surface (0–15 cm) of an experimental field under wheat at the Nuclear Institute for Agriculture and Biology, Faisallabad, Pakistan. Before use, the soil was air-dried and crushed to pass a 2-mm screen. Physicochemical analyses performed as described by Mulvaney and Kurtz (1982) gave the following results: pH (1:1), 7.4; organic C, 0.69%; total N, 0.07%; sand, 34%, silt, 52%.

To obtain different levels of ^{15}N-labelling in the microbial biomass, a preliminary study was conducted to determine the time required for complete immobilization of N applied as unlabelled $(NH_4)_2SO_4$, using added glucose as the C source. For this purpose, 100-g samples of soil were placed in 250-ml Erlenmeyer flasks and moistened to 60% water-holding capacity with a solution of $(NH_4)_2SO_4$ and glucose. Five different solutions were prepared so that the N-addition rate was 67, 133, 200, 267, or 333 μg g^{-1} soil, while in all cases, the C/N ratio was 30 with respect to the glucose. Assuming 40% efficiency in use of glucose C, the newly synthesized microbial biomass would have a C/N ratio of 12. The soils were incubated at 30 °C and sampled regularly to determine the time required for complete immobilization of added inorganic N; thereafter incubation was continued in order to study the onset of remineralization.

When the time required for complete immobilization of applied N had been determined, a similar study was conducted using ^{15}N-labelled $(NH_4)_2SO_4$ (2.746 atom % ^{15}N). After 106 h of incubation (until the applied ^{15}N was completely immobilized at all amendment levels), portions of the moist soil were extracted with 500 m*M* K_2SO_4 containing 5%, 10%, or 20% (v/v) $CHCl_3$ (time of shaking 1, 2, or 4 h). The resulting soil suspensions were filtered using Whatman no. 42 filter paper, and the extracts were analysed for total N by a semimicroKjeldahl procedure (Bremner and Mulvaney 1982). Isotope-ratio analyses were performed on the digested and distilled samples as described by Buresh et al. (1982), using a Nuclide Model 3-60-RMS mass spectrometer.

Additional portions of moist soil were exposed to $CHCl_3$ vapour for 24 h (Brookes et al. 1985b), and the fumigated soils were extracted with 500 m*M* K_2SO_4 (soil:extractant ratio 1:4; time of shaking 0.5 h). The soil suspensions were filtered (Whatman no. 1), and the filtrates immediately analysed for total N and ^{15}N. The N released by $CHCl_3$ fumigation (the chloroform fumigation-extraction method estimate of biomass N) was taken as the difference between N extracted with 500 m*M* K_2SO_4 from fumigated and unfumigated soil, as described previously.

All measurements were made in triplicate on moist samples. The results are expressed on an oven-dry soil basis.

Results and discussion

The immobilization of applied N was rapid and slow at low and high rates, respectively (Fig. 1). The time required for complete immobilization of N varied from

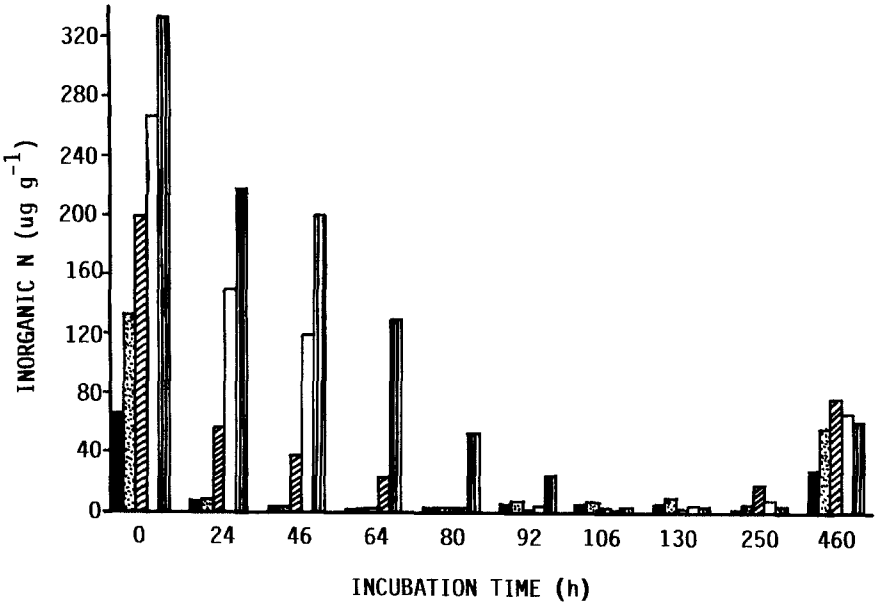

Fig. 1. Effect of incubation time on immobilization of applied ^{15}N as affected by the rate of addition of N (and glucose). The C/N ratio in all cases was 30. See also Azam et al. (1988b) ■ 67; ⊠ 133; ▨ 200; □ 267; ▥ 333 (μg g^{-1})

as little as 24 h with the two lower rates (66 and 133 μg N g^{-1}) to 106 h for the higher rates (C/N ratio of the amendment 30 in each case). Remineralization of N commenced at approximately 250 h for all amendment levels. These results suggest that, at the higher application rates, the applied N exceeded the demands of the prevailing microbial population. Although the added glucose would have been quickly metabolized at the lower application rates, with complete immobilization of applied N [half life of approximately 0.19 days (Voroney and Paul 1984)], net mineralization did not occur immediately (Fig. 1), suggesting internal cycling of N within the soil microbial population. This internal cycling would favour the development of a relatively more complex and diverse (taxonomically advanced) microbial population, and would be expected to result in reduced mineralization of the applied ^{15}N.

Depending upon the extraction method, 2.5% – 7.6% of the native soil N was extractable (Table 1). The fumigation-extraction method was always less effective than the extraction method in extracting soil N. With both techniques, extractable N increased consistently with an increase in the amount of N applied. One explanation for this effect is that the soil amendment caused a so-called priming effect, the magnitude of which increased with an increase in the quantity of material added. Although the occurrence and extent of priming has long been a subject of controversy, with both positive and negative effects having been reported following addition of C and/or N to soil (Jenkinson 1966; Jansson 1971; Olson 1980; Azam et al. 1988a), there is reason to believe that small priming effects could occur when a readily available C substrate is applied (glucose in the present study), because soil microorganisms react to additions of energy-rich materials (Jansson 1971), thereby affecting mineralization of native soil-organic matter.

Jansson (1971) and Hauck and Bremner (1976) suggested that the so-called stimulated mineralization of native soil N following addition of fertilizer N is a phenomenon of mineralization-immobilization turnover and is a result of net mineralization and accumulation of non-tagged N in a newly established inorganic N pool. The larger the pool, the more substantial will be the turnover, and the greater will be the contribution of native soil N to this pool. This, together with a possible priming effect from the added glucose, could account for our finding that there was an increase in the amount of native soil N extracted at the higher N-addition rates.

The percentage of the extracted N derived from the applied ^{15}N increased consistently and significantly with an increase in the amount of N (and glucose) added, irrespective of the extraction method (Table 2). Significant differences were observed between extraction procedures at each N-addition rate with respect to the contribution of applied ^{15}N.

In our study, the incubated soils were extracted at the point when immobilization of applied ^{15}N was maximal and before commencement of net mineralization. As a result, the ^{15}N recovered in the extracts can be assumed to have originated from the ^{15}N-labelled microbial biomass. Table 3 shows that recovery of applied ^{15}N in forms extractable by the fumigation-extraction method and the extraction method increased with an increase in the amount of N applied. This may have been due to rapid turnover of applied N at the lower addition rates, resulting in a quick succession of microbial populations, with incorporation of applied N into more complex and stable biomass constituents and metabolites. Because immobilization

Table 1. Percentage of native soil N extracted from fumigated and unfumigated soils with 500 m*M* K_2SO_4 and that extracted from unfumigated soil with 500 m*M* K_2SO_4 containing 5%, 10% and 20% $CHCl_3$

Extractant	Soil	Amount of applied N (μg g^{-1})				
		%				
		67	133	200	267	333
500 m*M* K_2SO_4	Fumigated	4.98	5.81	6.44	6.54	7.38
	Unfumigated	2.50	2.86	3.49	3.39	3.09
	CFEM-N[a]	2.48	2.95	2.96	3.15	4.31
500 m*M* K_2SO_4 + 5% $CHCl_3$	Unfumigated	3.23	2.99	5.11	5.65	5.33
+ 10% $CHCl_3$	Unfumigated	3.45	3.54	4.71	5.65	5.33
+ 20% $CHCl_3$	Unfumigated	3.49	3.68	5.06	6.29	7.31
LSD ($P = 0.05$)		0.98	1.01	1.35	1.61	2.52

[a] N released in soil due to $CHCl_3$ fumigation, i.e. the difference between N extracted from the fumigated and the unfumigated soil. The value thus obtained is used to calculate biomass N by the chloroform fumigation-extraction method (Brookes et al. 1985b)

Table 2. Percentage contribution of applied ^{15}N to the total N extracted from fumigated and unfumigated soils with 500 mM K_2SO_4 and from unfumigated soil with 500 mM K_2SO_4 containing 5%, 10%, and 20% $CHCl_3$

Extractant	Soil	Amount of applied N ($\mu g\ g^{-1}$)				
		%				
		67	133	200	267	333
500 mM K_2SO_4	Fumigated	36.09	51.11	63.64	74.21	78.86
	Unfumigated	36.24	53.75	65.64	75.95	80.85
	CFEM-N[a]	35.95	48.25	60.97	72.05	77.18
500 mM K_2SO_4 + 5% $CHCl_3$	Unfumigated	35.03	55.11	63.03	70.86	76.64
+ 10% $CHCl_3$	Unfumigated	31.65	50.06	65.65	76.93	80.91
+ 20% $CHCl_3$	Unfumigated	36.43	52.26	65.25	76.40	80.99
LSD ($P = 0.05$)		1.98	2.11	1.85	2.61	2.72

[a] See Table 1

Table 3. Percentage of applied ^{15}N extracted from fumigated and unfumigated soils with 500 mM K_2SO_4 and that extracted from unfumigated soil with 500 mM K_2SO_4 containing 5%, 10%, and 20% $CHCl_3$

Extractant	Soil	Amount of applied N ($\mu g\ g^{-1}$)				
		%				
		67	133	200	267	333
500 mM K_2SO_4	Fumigated	29.53	31.94	39.47	49.45	57.71
	Unfumigated	14.91	17.49	23.32	28.09	27.16
	CFEM-N[a]	14.62	14.45	16.15	21.36	30.55
500 mM K_2SO_4 + 5% $CHCl_3$	Unfumigated	18.29	19.31	30.50	36.05	36.70
+ 10% $CHCl_3$	Unfumigated	16.81	18.67	31.53	48.44	58.37
+ 20% $CHCl_3$	Unfumigated	21.00	21.14	33.25	53.50	65.29
LSD ($P = 0.05$)		0.98	1.01	1.35	1.61	2.52

[a] See Table 1

of the applied ^{15}N was completed in less time at the low addition rates compared with the high rates (Fig. 1), the microbial population was probably at a more advanced stage (all extractions were performed after 106 h of incubation). There is evidence that newly immobilized ^{15}N is redistributed among more complex fractions of the soil biomass upon completion of immobilization (Ladd et al. 1977). Ladd and Paul (1973) and McGill et al. (1975) found that the viability of newly formed microbial cells was low, and organic ^{15}N associated with them was incorporated into larger and more complex cells of a secondary population. Therefore, in studies where soil treated with easily degradable C compounds is extracted at the point of complete immobilization of applied inorganic N, more of the applied N is extractable (or mineralizable) and is derived from a simple, less heterogeneous and more labile microbial population; the reverse will be true for soils extracted long after the first signs of complete immobilization of added N. This hypothesis is supported by the previous work with the chloroform fumigation-incubation method. Voroney and Paul (1984) found that k_N values for a soil amended with glucose and NO_3^- increased with an increase in incubation time. A value of 0.2 was obtained after 1 day of incubation, and 0.3 after incubation for 42 days. This indicates that newly immobilized biomass N become less mineralizable with time, presumably because of an increase in the degree of complexity of the microbial population or/and probably the new cells became adsorbed by soil particles and are thus less available to $CHCl_3$ extraction.

Table 3 shows that 15% – 28% of the applied ^{15}N was recovered from the unfumigated soil by direct extraction with 500 mM K_2SO_4. This ^{15}N was probably derived from living microorganisms. The proportion of the biomass N extracted was increased substantially by fumigation, and also by the addition of $CHCl_3$ to the extractant (500 mM K_2SO_4), especially at the higher N-addition rates. The time allowed for extraction had, in general, no effect on the extractability of N. Therefore, only the results obtained for the 1-h ex-

Table 4. Extractability ratios for N extracted from fumigated and unfumigated soils with 500 m*M* K_2SO_4 and that extracted from unfumigated soil with 500 m*M* K_2SO_4 containing 5%, 10%, and 20% $CHCl_3$

Extractant	Soil	Amount of applied N ($\mu g\ g^{-1}$)				
		ER				
		67	133	200	267	333
500 m*M* K_2SO_4	Fumigated	5.93	5.49	6.13	7.56	7.82
	Unfumigated	5.97	6.11	6.69	8.29	8.85
	CFEM-N[a]	5.86	4.91	5.46	6.77	7.11
500 m*M* K_2SO_4 + 10% $CHCl_3$	Unfumigated	4.87	5.27	6.69	8.76	8.89

[a] See Table 1

traction have been presented. Higher values of extracted ^{15}N were obtained by the chloroform extraction method than by the chloroform fumigation-extraction method indicating that the former is probably more selective in extracting the newly immobilized biomass N. The difference in N extracted by the two methods increased with an increase in the amount of applied N. These results are in harmony with the suggestion of Brookes et al. (1985b) that the relationship between F_N (as determined by the chloroform fumigation-incubation method and the biomass N as determined by fumigation-extraction does not hold for soils that have recently received large quantities of substrate.

In the fumigation-extraction method biomass N is estimated by subtracting the total amount of N extracted from the unfumigated soil from the amount extracted following fumigation. Our results, however, indicate that estimates obtained in this way are low, and suggest that the N obtained by direct extraction of fumigated soil (i.e. without correction for the N extracted from unfumigated soil) is a better measure of biomass N. Our results also suggest that the extractability of biomass ^{15}N (Tables 3 and 4), as well as native soil N (Table 1), is increased when $CHCl_3$ is added directly to the extractant (500 m*M* K_2SO_4). Data obtained in this study show that the closest agreement with biomass N, as obtained by direct extraction of the fumigated soil, was achieved with a $CHCl_3$ concentration of 10% in the K_2SO_4 extracting solution and a 1-h shaking period (rest of the data not presented). Extraction of unfumigated soil by our proposed method (chloroform extraction method) is simpler, more rapid, and more convenient than the chloroform fumigation-extraction method while retaining the advantages of the latter. Moreover, the chloroform extraction method is more efficient and selective in extracting biomass N than the chloroform fumigation-extraction method, as revealed by the higher extractability ratios (% of applied ^{15}N extracted/% of native soil N extracted; He and Stevenson 1988) of the extracted N (Table 4). The data reported in Table 4 show that the extractability ratios were consistently greater than 1, which means that recently immobilized ^{15}N was more extractable than the native soil N.

Acknowledgments. We thank Emil Marcusiu for performing the isotope-ratio analyses. The investigation was supported by the Agricultural Experiment Station, College of Agriculture, University of Illinois at Urbana-Champaign and by the National Science Foundation (Grant No. INT-8503426).

References

Adams TM, Laughlin RJ (1981) The effects of agronomy on the carbon and nitrogen contained in the soil biomass. J Agric Res (Cambridge) 97:319–327

Azam F, Malik KA, Hussain F (1986) Microbial biomass and mineralization-immobilization of nitrogen in some agricultural soils. Biol Fertil Soils 2:157–163

Azam F, Mulvaney RL, Stevenson FJ (1988a) Quantification and potential availability of non-symbiotically fixed ^{15}N. Biol Fertil Soil 7:32–38

Azam F, Mulvaney RL, Stevenson FJ (1988b) Determination of in situ K_N by chloroform fumigation incubation method and mineralization of biomass under anaerobic conditions. Plant and Soil 111:87–93

Bremner JM, Mulvaney CS (1982) Total nitrogen. In: Page AL, Miller RH, Keeney DR (eds) Methods of soil analysis. ASA-ASSS, Madison, Wisc, pp 594–624

Brookes PC, Kragt JF, Powlson DS, Jenkinson DS (1985a) Chloroform fumigation and the release of soil nitrogen: The effects of fumigation time and temperature. Soil Biol Biochem 17:831–835

Brookes PC, Landman A, Pruden G, Kenkinson DS (1985b) Chloroform fumigation and the release of soil nitrogen: A rapid direct extraction method to measure microbial biomass nitrogen in soil. Soil Biol Biochem 17:837–842

Buresh RJ, Austin ER, Craswell ET (1982) Analytical methods in ^{15}N research. Fert Res 3:37–62

Hauck RD , Bremner JM (1976) Use of tracers for soil and fertilizer nitrogen research. Adv Agron 26:219–266

He Xin Tao, Stevenson FJ (1988) Revised formula for calculating extractability ratios of immobilized N in soils. Soil Biol Biochem (in press)

Jansson SL (1971) Use of ^{15}N in studies of soil nitrogen. In: McLaren AD, Skujins J (eds) Soil biochemistry, vol 2. Dekker, New York, pp 129–166

Jenkinson DS (1966) Studies on the decomposition of plant material in soil: II. Partial sterilization of soil and the soil biomass. J Soil Sci 17:113–137

Jenkinson DS (1976) The effects of biocidal treatments on metabolism in soil: IV. The decomposition of fumigated organisms in soil. Soil Biol Biochem 8:203–208

Jenkinson DS, Powlson DS (1976) The effects of biocidal treatments on metabolism in soil: V. A method for measuring soil biomass. Soil Biol Biochem 8:209–213

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

Ladd JN, Parsons JW, Amato M (1977) Studies of nitrogen immobilization and mineralization in calcareous soils: II. Mineralization of immobilized nitrogen from soil fractions of different particle size and density. Soil Biol Biochem 9:319–325

McGill WB, Shields JA, Paul EA (1975) Relation between carbon and nitrogen turnover in soil organic fractions of microbial origin. Soil Biol Biochem 7:57–63

Mulvaney RL, Kurtz LT (1982) A new method for determination of ^{15}N-labelled nitrous oxide. Soil Sci Soc Am J 46:1178–1184

Olson RV (1980) Fate of tagged nitrogen fertilizer applied to irrigated corn. Soil Sci Soc Am J 44:514–517

Voroney RP, Paul EA (1984) Determination of K_C and K_N in situ for calibration of the chloroform fumigation-incubation method. Soil Biol Biochem 16:9–14

Received February 23, 1988