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FACTORS INFLUENCING THE STABILITY OF LABELLED MICROBIAL MATERIALS IN SOILS

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Summary—The effect of freshly added substrate on carbon turnover of a microbial population and the priming action on stabilized soil organic constituents were investigated in the laboratory. ^{13}C-labelled glucose, NH_4NO_3, or both were added to samples of a Brown Chernozemic soil which had been initially amended with ^{14}C-glucose and incubated 2 months under field conditions. At the end of 14 days laboratory incubation, 39 per cent and 33 per cent of the ^{13}C had been respired as CO_2 from the glucose and glucose plus NH_4NO_3 treatments, respectively. These two treatments resulted in a marked priming of native ^{12}C during the second and third days of incubation and a second priming peak during the fifth day. In contrast, there was only a small priming action of the ^{14}C-labelled materials. Addition of NH_4NO_3 by itself had no effect on the amount of ^{12}C or ^{14}C respired.

Appreciable amounts of ^{14}C were mineralized following treatments known to partially sterilize soil. Freezing and thawing was more effective than wetting and drying, but less effective than $CHCl_3$ vapour in releasing stabilized ^{14}C materials. The amount of labelled-^{14}C mineralized during incubation after treatment with chloroform vapour was greater than could be accounted for by the decrease in soil biomass.

INTRODUCTION

The microbial biomass present in the surface 10 cm of grassland soil is estimated to be approximately 70 g/m^2 (Clark and Paul, 1970). A knowledge of the stability of microbial tissue is essential to assess its contribution to soil organic matter formation and nutrient cycling. The use of tagged microbial tissue in soil organic matter research was established by Jansson (1960) who reported that labelled tissue decomposed at a markedly decreasing rate and more slowly than the initial glucose amendment. Mayaudon and Simonart (1963), studying the humification of labelled tissue, reported that mycelium of the fungus *Aspergillus* exhibited greater stability in soil than cell walls of the bacterium *Azotobacter*.

The stability of soil organic matter also has been investigated after subjecting soil to wetting and drying, to freezing and thawing or exposure to organic solvent vapours. In many soils each successive re-wetting of a dry soil is accomplished by a flush of decomposition until the organic matter reaches some relatively stable form (Birch and Friend, 1961). This flush has been attributed to the effects of drying on the water-soluble material (Birch, 1959) and on the indigenous microbial population (Stevenson, 1956). Partial sterilization of moist soil with ether or chloroform resulted in successive flushes of CO_2 production in the absence of any enhanced solution of organic materials as occurs after drying. Consequently, it was postulated that part of each successive surge of CO_2 was due to the developing microbial population utilizing, as substrate, microorganisms killed by the vapour treatment (Birch, 1959). Similar conclusions were reached by Jenkinson (1966a) who observed that the specific activity of labelled C mineralized decreased after successive treatments with chloroform vapour.

Studies on the effects of freezing and thawing have generally shown less decomposition of organic matter and less destruction to bacteria after freezing than after drying (Soulides and Allison, 1961). Mack (1963) reported that although freezing increased the initial rate of organic matter decomposition, it had little effect on the total amount decomposed over the entire incubation period. He also observed that freezing caused a reduction in the number of viable fungi although neither freezing nor drying affected bacterial numbers. Other workers observed that only a small decrease in microbial numbers occurred when the soil was first frozen in contrast to a marked decrease in numbers when the frozen soil was thawed (Campbell *et al.*, 1970).

Accelerated decomposition of native soil organic matter by the microbial populations after the addition of readily available substrate is described as a priming action (Jenkinson, 1966b). Isotopic techniques permitted measurements of the proportions of respired CO_2 derived from the added substrate or from the soil.

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Broadbent and Norman (1946) showed that mineralization of soil organic matter was greatly accelerated by the addition of ^{13}C-labelled Sudan grass. Subsequent investigations with labelled glucose (Chahal and Wagner, 1965), labelled plant material or its components (Mortenson, 1963; Sorensen, 1963; Sauerbeck, 1966) have established that addition of organic material to soil results in some degree of priming action. However, the effects are short lived and small in comparison to the amounts of native organic matter present (Jenkinson, 1971). Jansson (1960) found that sequential additions of unlabelled glucose to soil previously incubated with ^{14}C-labelled glucose did not cause priming of the labelled material present.

In this study, the priming action and the effect of freshly added substrate labelled with ^{13}C were investigated using soil samples which had been initially amended with ^{14}C and incubated under field conditions (Shields *et al.*, 1973). The labelled field soil was also subjected to a range of physical and chemical treatments in an attempt to elucidate the mechanisms responsible for the stabilization of labelled microbial material.

MATERIALS AND METHODS

The Sceptre soil samples used were those which had been initially amended with uniformly labelled ^{14}C-glucose and incubated under field conditions (Shields *et al.*, 1973).

Soil respiration and microbial measurements

Samples of labelled soil (41·4 mg ^{14}C/100 g soil) removed from the field 56 days after amendment and equivalent to 100 g of dry soil, were placed in 250 ml Erlenmeyer flasks and amended with the following treatments in triplicate:

1. Control with no addition.
2. One hundred milligrams C as uniformly labelled ^{13}C-glucose in solution (2·65 atom % excess).
3. Four milligrams N as NH_4NO_3 in solution.
4. ^{13}C-glucose + NH_4NO_3 solution containing (2) + (3).

The moisture content of all samples was adjusted to 33 per cent (80 per cent field capacity) and incubated at 25 C. Moisture-saturated, CO_2-free air was passed continuously through the flasks and respired CO_2 collected in 0·2 *N* NaOH in a glass flask and a tower assembly filled with 3 mm glass beads. The towers were changed daily and duplicate 1 ml NaOH samples taken for radioactivity determinations. The remaining NaOH was titrated after precipitating the absorbed CO_2 as $BaCO_3$. The precipitate was filtered through fiberglass, dried at 60 C and stored for ^{13}C analysis on the mass spectrometer.

For microbial counts, samples (150 g oven dry basis) from the unlabelled, glucose-amended, field soil were placed in 400 ml beakers and treated (in duplicate) as above but with unlabelled glucose where appropriate. After adjusting the moisture content to 33 per cent, the soils were incubated at 25 C in a large desiccator supplied continuously with fresh moist air and sampled at intervals. Samples treated for soil respiration and for microbial measurements were all incubated within the same controlled environment.

Physical treatments

Samples of labelled soil (36·5 mg ^{14}C/100 g soil) removed from the field 90 days after amendment and equivalent to 100 g dry wt. were placed in 250 ml Erlenmeyer flasks of known weight. The moisture content was adjusted to 33 per cent before the following treatments:

1. Control with no addition.
2. Wetting and drying: samples were placed in a forced-air oven at 25 C for 48 h, reducing the moisture content to 5 per cent. The moisture content was then restored to 33 per cent by addition of water and the samples incubated at 25 C for 48 h. This cycle was repeated five times.
3. Freezing and thawing: samples were placed in a refrigerated bath at 25 C. The temperature of the bath was gradually lowered to −7 C over a period of 4 h and held constant for 20 h. The bath temperature was then raised to 25 C over a 4 h interval and maintained for 20 h. This cycle was repeated 12 times.
4. Wetting and drying plus freezing and thawing: samples were subjected to one wetting and drying cycle as described in (2), followed by one freezing thawing cycle as described in (3). This entire sequence was repeated five times.
5. Autoclaved at 121 C for 1 h, then re-inoculated with 1% inoculum of untreated field soil.
6. Chloroform vapour: samples were placed in a chamber containing a small beaker of redistilled $CHCl_3$ and a lining of sorbent paper moistened with $CHCl_3$. The chamber was evacuated until the $CHCl_3$ began to boil and placed in the dark at 25 C for 48 h. After removal of the $CHCl_3$ and the paper, the chamber was evacuated four times for 15 min intervals to remove $CHCl_3$ vapour adsorbed to the soil. The samples were then re-inoculated with 1% inoculum of the untreated field soil.

After each treatment, the moisture content was adjusted to 33 per cent and the samples incubated at 25 C for 14 days. The control soil was sampled before and after the incubation period.

Measurements of microbial material

The techniques for determination of plate count and direct count bacterial numbers have been described previously (Shields *et al.*, 1973).

Analytical methods

The determination of carbon and radioactivity of soil samples were as described by Shields and Paul (1973). An Atlas Model GD 150 mass spectrometer was used for the determination of ^{13}C. Analytical procedures and calculations for ^{13}C are given by Martel (1971).

Table 1. Total C respired from labelled Sceptre soil during laboratory incubation

Day	Treatment			
	Control	NH_4NO_3	Glucose	Glucose plus NH_4NO_3
	mg C/100 g soil per day			
1	4·5	4·6	36·2	25·8
2	3·9	4·1	40·5	40·1
3	4·8	4·3	21·2	22·5
4	5·4	4·6	4·4	3·9
5	4·8	5·6	10·9	11·3
6	5·3	5·1	9·4	9·7
7	5·1	5·2	8·1	6·5
8	5·5	*	5·5	7·3
9	5·2		4·7	6·5
10	5·6		5·8	6·1
11	5·1		5·8	6·2
12	5·1		5·8	6·2
13	4·2		4·7	5·4
14	4·4		3·9	5·0
Total	68·9		166·9	162·5

* Respiration terminated after 7 days.

RESULTS

The total C respired by the control soil during the incubation period ranged from 4 to 6 mg C/100 g soil per day (Table 1). Respiration of soil amended with NH_4NO_3 did not differ from that of the control during the first week; further measurements were not made. The rate of respiration of samples amended with ^{13}C-glucose or ^{13}C-glucose plus NH_4NO_3 increased rapidly to a maximum of 40 mg C/100 g soil per day on day 2 before declining to that of the control soil on day 4. The rate increased again to twice that of the control in days 5 and 6 before stabilizing at the control level. After 1 week, the total C evolved from soil treated with glucose exceeded that from soil treated with glucose plus NH_4NO_3 by 10 mg; this difference was not apparent after 14 days.

Addition of ^{13}C-glucose to soil containing a ^{14}C-labelled population and metabolites made it possible to measure the effect of additional substrate on the stabilized ^{14}C-material and on the native soil organic matter (^{12}C). The components (^{12}C, ^{13}C, ^{14}C) of the total C respired (Fig. 1) from samples amended with ^{13}C-glucose or ^{13}C-glucose plus NH_4NO_3 show a similar pattern. Added ^{13}C-glucose was rapidly degraded by the soil population in the presence and absence of added N during the first 3 days. After this, the rate declined to about 0·4 mg C/day per 100 g soil. This is comparable with the rates of added ^{14}C mineralized after the first week of incubation during the previous field experiment (Shields *et al.*, 1973). On day 1, labelled ^{13}C respired from glucose-treated soil exceeded that of soil treated with glucose plus

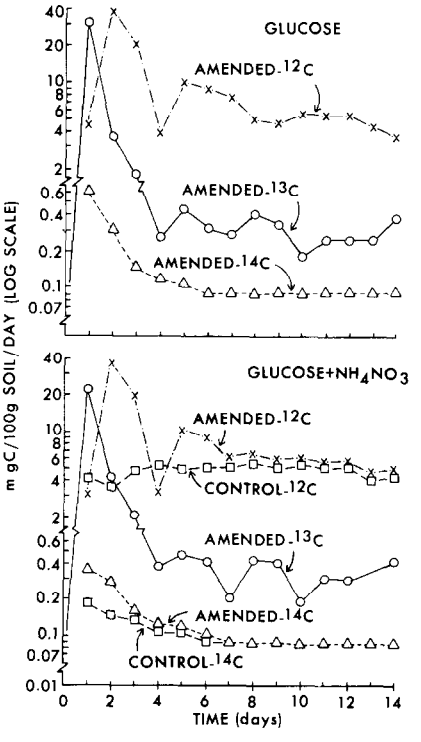

Fig. 1. Carbon isotopes respired from previously labelled Sceptre field soil after amendment with ^{13}C-glucose or ^{13}C-glucose plus NH_4NO_3 in the laboratory.

NH_4NO_3 by 8 mg C and represented the major difference between treatments. The amount of labelled ^{13}C mineralized in the glucose-amended soil was 37 per cent after 7 days and 39 per cent after 14 days. The corresponding values for soil amended with glucose plus NH_4NO_3 were 31 per cent after 7 days and 33 per cent after 14 days, indicating a greater stabilization of the added glucose-C in the presence of added N. Mayaudon (1971), using radiorespirometric techniques to analyze the glycolytic activity of fresh meadow soil, reported that one-quarter of the glucose-3, 4-^{14}C (added at a concentration of 3·6 mg/100 g soil) was mineralized after 80 min by which time the system had reached equilibrium. Shields *et al.* (1973) observed that 40 and 44 per cent of the added glucose ^{14}C was evolved by Sceptre field soil after 7 and 14 days, respectively. Results of the present study indicate that 60–70 per cent of the added carbon remained in the soil as synthesized microbial tissue or its metabolites.

The upper curves in both parts of Fig. 1 show that the rates of ^{12}C respired from both glucose amendments were highest (5·9 × control) during the second and third day with only minor deviations from that of the control soil occurring thereafter. The rapid decline in ^{12}C evolved from the amended soils on day 4 is considered to be significant and cannot be attributed to malfunction of the respiration apparatus since this effect was absent from the control samples. Double peaks in the rate of soil C evolved following amendment with glucose were also reported by Keefer and Mortenson (1963). A similar pattern of ^{12}C mineralization was observed during the laboratory incubation of Dark Brown, fine sandy loam amended with ^{14}C-glucose (J. N. Ladd, *personal communication*). The amount of inorganic ^{13}C measured (1·3 mg C/100 g soil) on termination of the incubation period (data not shown) was similar to the small quantities of labelled inorganic C reported by Shields *et al.* (1973) after addition of ^{14}C-glucose to Sceptre field soil.

The ^{14}C respired from amended soils did not exceed the amount respired by the control soil after day 4 (Fig. 1). During the first 3 days, the increase over control ranged from a total of 0·2 0·6 mg ^{14}C indicating only a small priming action on the labelled metabolites. Addition of NH_4NO_3 by itself had no effect on the amount of ^{14}C respired.

Examination of Table 2 shows that the most rapid decomposition of added glucose (^{13}C + ^{14}C) in either treatment occurred during day 1. The maximum rate of breakdown of native soil organic matter (^{12}C) coupled with the lower rates of glucose C respiration resulted in a marked priming action on the native soil C during the second and third day of incubation and to a lesser extent from days 5 to 7. On termination of the experiment, the cumulative priming action observed in the glucose and glucose plus NH_4NO_3 treatments was 57 and 60 mg C/100 g soil, respectively. The priming action therefore doubled the ^{12}C respired during the 14-day incubation period. Apparently, the ^{12}C substrate attacked by the active microbial popula-

Table 2. Origin of C respired from ^{14}C-labelled Sceptre soil amended in the laboratory

	Treatment					
	^{13}C-glucose			^{13}C-glucose + NH_4NO_3		
	Respired C derived from			Respired C derived from		
Day	Glucose (^{13}C + ^{14}C)	Soil (^{12}C)	Priming action*	Glucose (^{13}C + ^{14}C)	Soil (^{12}C)	Priming action*
	mgC/100 g soil per day					
1	31·7	4·5	0·0	22·7	3·1	−1·4
2	4·1	36·4	32·5	4·4	35·7	31·8
3	1·1	20·1	15·3	2·4	20·1	15·3
4	0·4	4·0	−1·4	0·5	3·4	−2·0
5	0·6	10·3	5·5	0·6	10·7	5·9
6	0·4	9·0	3·7	0·5	9·2	3·9
7	0·4	7·7	2·6	0·3	6·2	1·1
8	0·5	5·0	−0·5	0·5	6·8	1·3
9	0·4	4·3	−0·9	0·5	6·0	1·2
10	0·3	5·5	−0·1	0·3	5·8	0·2
11	0·4	5·4	0·3	0·4	5·8	0·7
12	0·3	5·5	0·4	0·4	5·8	0·7
13	0·3	4·4	0·2	0·5	4·9	0·7
14	0·5	3·4	−1·0	0·5	4·5	0·1
			+56·6			+59·5

* Soil carbon respired from amended soil minus carbon respired from control soil as shown in Table 1.

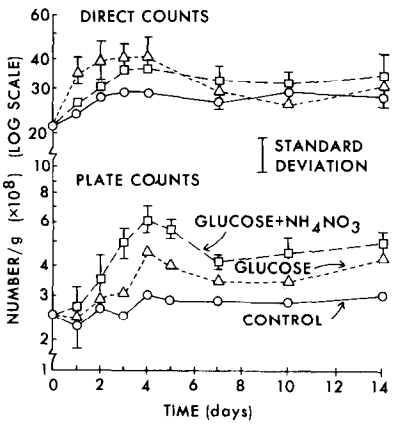

Fig. 2. Viable and total bacterial numbers in Sceptre soil during laboratory incubation period.

tion present during the period of maximum priming was rapidly depleted by day 4. It is postulated that following a short period required for adjustment of extracellular enzyme systems characteristic of the existing populations or for a qualitative shift in the populations, the microflora were then able to attack another form of native soil organic matter as shown by the ^{12}C respired from days 5 to 7.

Changes in microbial numbers are given in Fig. 2. Numbers of plate count bacteria for soil treated with glucose and with glucose plus NH_4NO_3, increased two-fold after 4 days, before declining slowly during the remainder of the experimental period. The direct count of bacteria in amended soils also doubled before decreasing to the level of the control soil. The higher numbers of plate count organisms in the glucose plus NH_4NO_3 soil corresponded to a greater efficiency of C utilization; this was not reflected in the direct count data. The plate count numbers, and the patterns of change following amendment, were similar to those observed following amendment of similar soil under field conditions (Shields *et al.*, 1973), but total bacterial numbers by the direct count technique showed a higher degree of stability under the constant abiotic conditions present in the laboratory than under field conditions.

In contrast to the small amount of ^{14}C mineralized following amendment with ^{13}C-glucose, appreciable amounts were mineralized following treatments known to partially or completely sterilize soil (Table 3). Treatments simulating natural physical processes showed that labelled C mineralized after freezing and thawing (16 per cent of residual C) was double that from wetting and drying, or wetting and drying combined with freezing and thawing. The similarity between the two latter treatments suggests that the wetting and drying sequence when in combination with freezing thawing tended to reduce the labelled C mineralized as a result of the freezing and thawing. Maximum mineralization occurred after treatment with $CHCl_3$ with more than three-quarters of the total evolved (30 per cent) occurring within 7 days of re-inoculation. Only a small amount of labelled C was mineralized during the second week of incubation.

Treatment with $CHCl_3$ reduced numbers of plate count bacteria to less than 2×10^5 organisms/g, or 0·1 per cent of the population of untreated soil (Fig. 3). Following re-inoculation, plate count numbers increased rapidly to a maximum of 6×10^8 organisms/g

Table 3. Labelled C mineralized from previously amended Sceptre soil* after partial or complete sterilization

Treatment	Days of incubation	mg C/100 g soil		% of residual-^{14}C mineralized
		Remaining	Mineralized	
Control	0	36·5	—	—
	14	35·2	1·3	3·5
Wetting and drying	14	33·8	2·7	7·4
Wetting and drying plus freezing and thawing	14	33·6	2·9	7·9
Freezing and thawing	14	31·0	5·5	15·0
Autoclave	14	29·5	7·0	19·2
Chloroform	0†	36·5	—	—
	2	29·9	6·6	18·0
	4	28·5	8·0	21·9
	7	28·0	8·5	23·3
	9	27·6	8·9	24·4
	14	26·4	10·1	27·7
	21	25·7	10·8	29·6

* Removed from the field 90 days after adding ^{14}C-labelled glucose (93·7 mg C/100 g soil).
† Sampled immediately after treatment with chloroform before reinoculating and incubating.

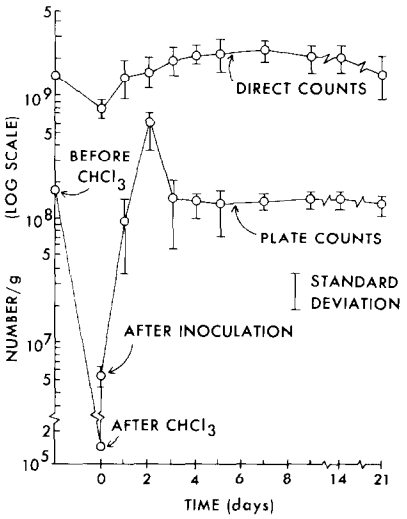

Fig. 3. Bacterial numbers in Sceptre soil before and after treatment with chloroform.

after 2 days, before declining to numbers similar to those present before treatment. This rapid increase in viable numbers coincided with the high rate of labelled C mineralized during the same period (Table 3). Direct counts of bacteria also declined after treatment with $CHCl_3$ (Fig. 3). Following re-inoculation, numbers increased steadily to a maximum of $2{\cdot}5 \times 10^9$ organisms g after 7 days before declining to pre-treatment numbers after 2 weeks.

DISCUSSION

The data presented in this paper show that ^{13}C-labelled glucose with or without nitrogen added to ^{14}C-labelled Sceptre soil was rapidly metabolized during the first 3 days of incubation. The newly synthesized microbial material was also subject to decomposition, but at a greatly reduced rate. Shields and Paul (1973), following the decomposition of labelled straw through soil organic fractions, found that biological products arising from the rapid initial attack on easily degradable plant components exhibited a high degree of stability in Sceptre soil under field conditions. Investigation of the turnover of doubly labelled microbial populations at the Sceptre site indicated a half-life of 4 days for the added glucose compared with 6 months for the synthesized microbial tissue and metabolites (Shields *et al.*, 1973).

Jansson (1960) followed the decomposition of labelled glucose in soil which subsequently received three successive amendments with unlabelled glucose at weekly intervals. Fifty-seven per cent of the labelled glucose was mineralized during the first week of incubation. Subsequently, 7 per cent of the added tagged C was evolved after the first unlabelled amendment, 4 per cent after the second and 3 per cent after the third. He concluded that decomposition of the earlier additions were unaffected by the later ones. In the present study, addition of ^{13}C-glucose resulted in less than 1 per cent mineralization (in excess of control) of the radioactive microbial materials formed from the ^{14}C-glucose added to the soil 2 months previously (Shields *et al.*, 1973). Evidently, stabilization of the relatively large amount of ^{14}C-labelled microbial material observed under field conditions was due to lack of neither energy or nitrogen, nor was is susceptible to priming. Mineralization of appreciable amounts of this material occurred only after treatments known to kill the microbial population.

Mack (1963) reported that rapid freezing and short-term drying resulted in a marked increase in the subsequent mineralization of C and N in soil organic matter. Drying had no effect on the number of fungi or bacteria whereas freezing decreased the number of viable fungi. Biederbeck and Campbell (1971) also reported that freezing caused only a small reduction in microbial numbers. However, thawing at fluctuating temperatures was lethal, particularly in recently cropped soil. In the present study, appreciably more labelled C was mineralized during incubation following freezing and thawing than after wetting and drying, suggesting much of the labelled biomass remaining in the soil was killed during the freeze–thaw cycles. Alternatively, it may be argued that the physical disruption of the soil aggregates accompanying freezing and thawing exposed a larger surface of clay absorbed metabolites to microbial attack. It is highly probable that part of the labelled C mineralized by these treatments was derived from both the biomass and metabolite sources.

Jenkinson (1966a), exposing labelled soil samples to a range of treatments which partially or completely sterilize soil, reported that a small heavily-labelled organic fraction was rendered decomposable. He postulated that this fraction was the soil biomass. Table 3 indicates that labelled microbial material mineralized after $CHCl_3$ was double that released from physical processes. This was attributed to solubilization of lipid components of the cytoplasmic membrane and cell wall by $CHCl_3$ vapour resulting in lysis of the cells which provided substrate for the re-inoculated population. Plate count measurements, together with determination of labelled C remaining in the soil, indicated that most of the material was mineralized by an extremely active viable microbial population.

According to Jenkinson (1966a), biomass can be determined from the amount of CO_2 evolved after $CHCl_3$ treatment. Table 3 shows that following exposure to $CHCl_3$ vapour, 8·5 mg ^{14}C/100 g soil were mineralized from the re-inoculated soil during the first week of incubation. Using Jenkinson's values for CO_2 evolved upon attack of labelled population (30 per cent), then $8{\cdot}5/0{\cdot}30 = 28$ mg C/100 g soil or three-quarters of the labelled C present at the time of $CHCl_3$ treatment was rendered available by the treatment. If the biomass were the exclusive source of the evolved C, as suggested by Jenkinson (1966a), then only about one-quarter of the labelled C in the soil

before treatment was represented by non-living material.

McGill *et al.* (1973) concluded that 10 to 30 per cent of the total pool of labelled microbial material resulting from growth on ^{14}C-acetate could be accounted for as identifiable biomass. The rest was assumed to be present as extracellular metabolites or lytic products stabilized in the soil system.

Microscopic measurement of the microbial population in the field study that provided the soil for this investigation indicated that on day 7 the net increase of identifiable bacterial and fungal biomass was 192 μg C/g, whereas the total residue of labelled C was 559 μg C/g soil (Shields *et al.*, 1973). After 3 months in the field when the soil was sampled for this study the residual labelled carbon accounted for 370 μg C/g soil and a total of 570 μg C/g could be accounted for as identifiable microbial tissue (Shields *et al.*, 1973). The population at this time was growing primarily on soil carbon for the data in Fig. 1 show that the ^{12}C content of the respired CO_2 of the control soil was 20–40 times that of the ^{14}C.

The above indicates that much of the population was non-labelled and the flush of ^{14}C on physical treatment cannot be ascribed strictly to biomass. It is possible that a portion of the biomass was not identified by the microscopic techniques employed and we support Jenkinson's (1966a) concept that $CHCl_3$ treatment releases susceptible biomass carbon which is normally resistant to decomposition. However, much of the ^{14}C also must persist in non-identifiable microbial breakdown products which are stabilized in the soil until physical treatment or $CHCl_3$ made them susceptible to attack.

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