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Measurement of bacterial growth rates in soil

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Abstract

Measurements of the average growth rates of soil bacteria from a soil under three treatments; a conventional high input corn soybean rotation, a reversion to a native successional community (4 years) and a never ploughed grassland, were made by two methods. The specific rates of synthesis of bacterial DNA were determined from the specific activities of the DNA precursor, deoxythymidine triphosphate (dTTP), and purified bacterial community DNA after ^{3}H thymidine incorporation. These rates were compared with the specific respiration rates of the soil microbiota determined from the initial rate of C mineralization and the microbial biomass C. We show how these two measurements can be used to estimate values for microbial growth yield and maintenance coefficients in soil. Both methods ranked the soil treatments in the same order. The reversion treatment had the highest thymidine incorporation and specific respiration rates and the corn soybean rotation the lowest. The grassland soil had the highest microbial biomass but an intermediate growth rate. Based on thymidine incorporation the doubling time of the bacterial biomass at 25°C was 160 days in soil from the corn soybean rotation and 107 days in the reversion treatment. Fractionation of the ^{3}H-labelled DNA according to its %guanine+cytosine content showed growth of soil bacteria to be widespread among bacterial genera and not confined to a few active species. The soil bacterial growth rates found here are much lower than those found in some other studies using thymidine incorporation but are in agreement with annual C budgets for the soils.

Keywords: %G+C; Microbial growth; Soil; Specific respiration; Thymidine incorporation

1. Introduction

Knowledge of the growth rates of microorganisms is fundamental to an understanding of their ecology. The mineralisation-immobilisation reactions involved in the growth and maintenance of soil microorganisms determine the availability of plant nutrients such as nitrogen and to a large extent phosphorus and sulfur. These nutrients largely control ecosystem functioning and agricultural soil productivity. Estimates of the average growth rate of the soil microbiota can be made from carbon (C) turnover if values for maintenance and growth yield coefficients are known or assumed. These two parameters are difficult to measure in realistic soil environments (Payne and Wiebe, 1978) and are frequently combined as an overall efficiency term. As maintenance is independent of growth its inclusion in a yield efficiency parameter makes measurement of growth rate from C turnover

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data impossible. Independent measurements of growth rate, maintenance coefficient and yield efficiency in soil are required for an understanding of the relationship between substrate utilization, C mineralization and biomass formation.

^{3}H thymidine uptake and incorporation into DNA is a powerful and exact means of directly measuring replication rates in bacteria providing the limiting requirements and underlying assumptions are understood (Fuhrman and Azam, 1982; Pollard and Moriarty, 1984). The requirements fall into two groups, the first group is concerned with the methods used to estimate thymidine uptake and incorporation and includes:
(1) Measurement of the specific activity of deoxythymidine triphosphate (dTTP), the precursor to DNA synthesis, by an isotope dilution method (Forsdyke, 1971); this is necessary because the extent of dilution of this pool by unlabelled dTTP is unknown.
(2) Measurement of the specific activity of the synthesized DNA, excluding products formed from catabolism of the added ^{3}H thymidine (Brittain and Karl, 1990).
(3) Ensuring that DNA synthesis is the rate limiting step for thymidine incorporation.
(4) Disturbance of the sample during measurement may alter the growth rate (Moriarty and Pollard, 1990; Findlay et al., 1990).
A second group of problems arises in natural microbial communities and includes: (1) errors resulting from the failure of some bacteria to incorporate thymidine into DNA (Pollard and Moriarty, 1984; Jeffrey and Paul, 1990; Christensen, 1993); (2) failure to extract a representative sample of the bacterial DNA from the soil; (3) co-extraction of DNA from sources other than living bacteria.

The advantage of using DNA as a labeling target is two-fold. Firstly, DNA replication is a fundamental attribute of growth and is in this sense absolute. The specific growth rate can be determined directly from the specific activities of dTTP and DNA. Much of the controversy associated with the use of ^{3}H thymidine uptake as a method of measuring microbial growth has been concerned with the use of factors for the conversion of ^{3}H thymidine incorporation rates into rates of bacterial productivity (e.g. Moriarty, 1986; Kirchman and Hoch, 1988; Chrost et al., 1988; Coveney and Wetzel, 1988; Bååth and Johansson, 1990). The conversion factors vary widely for different species within a microbial community (Christensen, 1993), this means that the conversion factor is very system specific. However, provided that the conversion factor is valid, this approach has the advantage of accounting for the presence of non-incorporating organisms in the microbial community.

The thymidine uptake procedure has been refined to avoid many of the errors which can arise from catabolism of ^{3}H thymidine and the lack of direct measurement of the specific activities of precursor and product. We have applied the refined methodology to the measurement of replication rates in soils from a long term ecological research (LTER) site and compared them with estimates derived from C turnover measurements.

A second advantage of using DNA as a target for labeling studies is that the base sequence of the DNA is conserved. This presents the potential to sort or separate labelled DNA and to determine replication rates for particular components of the soil bacterial community. The ^{3}H thymidine-labelled DNA isolated from these soils have been examined for the distribution of label in DNA of different %G+C by fractionation of bisbenzamide-DNA complex on CsCl density gradients (Holben and Harris, 1991), this is one example of how the thymidine-labelled DNA can be sorted.

2. Materials and methods

2.1. Soils

Samples of the 0–10 cm layer were collected in August 1991 from a Kalamazoo series soil (Typic Hapludalfs, fine loamy, mixed, mesic) on treatment plots of a long term agroecology experiment at the Michigan State University, Kellog Biological Station, Battle Creek, MI. The treatments were: (1) corn/soybean rotation with conventional fertilization (110 kg ha^{-1} N as am-

monium nitrate), the crop was corn; (2) reversion to a native successional community on soil taken out of cultivation in 1988; (3) never tilled, grass field. Soils from Treatments 1 and 2 contained 0.9% organic C, 0.1% N and that from Treatment 3, 1.6% organic C and 0.15% N, pH on all treatments was 6.3. The soils were sieved moist to 2 mm and stored at 5°C until use (less than 2 weeks).

2.2. ^{3}H *(methyl)-thymidine*

The stock concentrate (248 GBq mmol^{-1}, sterile aqueous solution, 1 ml, New England Nuclear Products, Boston, MA) was diluted to 370 ml (0.403 μM) with sterile water and stored at 5°C until use.

2.3. *Soil slurry respiration*

To quickly and uniformly introduce added thymidine to the microbiota the soils were incubated as slurries. CO_2 evolution from the soil of Treatment 1 with and without the addition of the water or thymidine, was measured to test if slurry formation caused short-term changes in the activity of the soil microbial community. Tubes containing soil (10 g) were pre-incubated at 25°C for 24 h then flushed with air and capped with rubber serum stoppers. Samples (0.2 ml) of the headspace gas were removed at 10 min intervals in 1 ml syringes. The gas samples were stored for up to 2 h by inserting the needle into rubber stoppers. After 30 min incubation of the soil samples, 10 ml of water, thymidine (750 nM) or formaldehyde solution (0.1%) were added from a syringe through the serum cap with the removal of an equivalent volume of headspace gas. The soil and solution were mixed by vortex then incubated horizontally on a reciprocating shaker at 60 r.p.m. Control tubes had no additions to the soil and were not shaken. Headspace gas sampling was continued for a further 2 h. The CO_2 concentrations in the samples were measured on a gas chromatograph fitted with a Poropak Q column and thermal conductivity detector. A standard mixture of 1% CO_2 in air served as a calibration standard.

2.4. *Time course of* ^{3}H *thymidine uptake*

Slurries of replicate 10 g samples of soil in 30 ml centrifuge tubes were formed by addition of 10 ml ^{3}H thymidine solution (0.403 μM, 0.25 MBq nmol^{-1}). The tubes were incubated at 25°C on a reciprocating shaker (60 r.p.m.). ^{3}H thymidine uptake was terminated after 5, 30, 60, 90, 120 and 150 min by addition of excess ^{1}H thymidine (1 ml 750 mM) and rapid cooling to 0°C in an ice bath. The slurries were sedimented by centrifugation at 7800 *g* for 10 min at 5°C. The soil was washed by resuspending in 10 ml phosphate buffer (1.0 mM, pH 7.0) and sedimented again. The pellet was resuspended in phosphate buffer (10 ml, 1.0 mM, pH 7) before DNA extraction.

2.5. *Isotope dilution measurements*

The specific activity of the DNA precursor, deoxythymidine triphosphate (dTTP), was estimated by an isotope dilution method. This method uses a constant aliquot of labelled thymidine along with incremental dilutions of unlabelled thymidine to measure the reduction in specific activity of dTTP due to initial pools and any unrepressed de novo synthesis of thymidine. Forsdyke (1971) showed the relationship between the additions of unlabelled thymidine $(y+1)$, in dilution units, and the reciprocal of the radioactivity of the DNA $(1/x)$ to be

$$y+1=(1/x)n-p \qquad (1)$$

and showed that the slope (n) of the linear plot estimated the radioactivity of the DNA if intrinsic dilution of the dTTP pool was zero. Intrinsic dilution of the dTTP pool, either by already existing initial pools of thymidine or dTTP, or by unrepressed de novo synthesis, is estimated by the intercept p. The radioactivities x and n are equivalent to specific activities for unit mass. The reciprocal plot is useful for testing the linearity of the relationship, but in practice, values of n and p were obtained by non-linear regression using the model (Pollard and Moriarty, 1984)

$$x=\frac{n}{(p+y+1)} \quad (2)$$

Non-linear regression avoids errors inherent in the linearization of data using reciprocals.

Specific DNA synthesis (D) then follows from

$$D=\frac{nb}{Ta} \quad (3)$$

where b is the molecular weight of thymidine 5′ monophosphate (322.2), a is the mass fraction of thymidine 5′ monophosphate in DNA at 68% G+C (0.1576) and T is the specific activity of the labelled thymidine (Bq μmol^{-1}) The soil bacterial DNA replication rate (μ h^{-1}) was derived from D by

$$\mu=\ln\frac{1}{1-\frac{D}{t}} \quad (4)$$

which is a special case of the integrated form of the microbial growth equation where DNA at the end of the growth period is normalized to unity. When D is small it closely approximates μt because

$$\ln\frac{1}{1-D}\rightarrow D$$

Replicate 10 g soil samples were slurried with 10 ml of water containing 0.25 MBq ^{3}H thymidine at five incremental thymidine concentrations, from 0.40 to 2.2 μmol, representing dilution factors one to five of the thymidine content of the lowest dilution. The slurries were shaken (25°C, 60 r.p.m.) for 2 h. ^{3}H thymidine uptake was terminated as before and the soil samples washed once in phosphate buffer prior to DNA extraction.

2.6. Soil bacterial DNA extraction and purification

Soil bacterial DNA was extracted from the soils after ^{3}H thymidine incorporation by a minor modification of the direct lysis procedure of Holben (1993). DNA purification by isopycnic banding in CsCl gradients was performed in smaller (6 ml) centrifuge tubes, this reduced the dilution rate of impurities compared with that obtained in 18 ml centrifugation tubes but use of the smaller tubes enabled up to 40 samples to be centrifuged simultaneously, compared with only eight by the standard method. The procedure for recovery of the DNA from the CsCl density gradient was also modified to improve recovery. The DNA bands were removed from the CsCl gradients by piercing the wall of the tube with a syringe needle and withdrawing 1–2 ml. Ethidium bromide was removed from the DNA by extraction with a 3x volume of NaCl-saturated isopropanol; this was repeated five times. The DNA solution was then diluted to 5x the original (1–2 ml) volume with TE (10 mm tris.HCl 1 mm EDTA pH 8.0) in 30 ml glass centrifuge tubes and an equivalent volume (5–10 ml) of isopropanol added. The DNA was allowed to precipitate for 1 h at 0°C. The tubes were then centrifuged at 7800 g for 40 min at 5°C. The isopropanol was decanted, the tubes inverted and allowed to dry (about 1 h). The DNA was redissolved in 500 μl TE at 65°C for 1 h with frequent shaking. The solution was transferred to a 1 ml microfuge tube and mixed with 50 μl Na acetate (3M) and one volume of isopropanol. The microfuge tubes were centrifuged at 16 000 g for 15 min then decanted. The pellets were rinsed with 70% EtOH then 95% EtOH, allowed to dry, then redissolved in 500 μl TE at 65°C.

2.7. HPLC separation of nucleotides from ^{3}H DNA

Aliquots of ^{3}H thymidine-labelled DNA (30 μg) from the soils treated with the highest specific activity thymidine (0.25 MBq nmol^{-1}) were digested to monomers with nuclease P1 (Tamaoka and Komagata, 1984). The DNA was dissolved in 100 μl water, denatured at 100°C for 5 min and then cooled rapidly in ice. The denatured DNA solution was mixed with 50 μl of nuclease P1 solution (0.1 mg in 1 ml 40 mM sodium acetate buffer containing 2 mM zinc sulfate, pH 5.3) and incubated at 50°C for 2 h. The digest was diluted to 300 μl and deoxynucleoside monophosphates were chromatographically sep-

arated on a 250 mm × 4.6 mm Spherisorb S5 SAX ion exchange column (Anspec Co, Ann Arbor, MI) using 50 mM KH_2PO_4/H_3PO_4 pH 3.0 at a flow rate of 1 ml min^{-1} as eluent. Peak areas and retention times of nucleoside monophosphates were compared with those of standard samples (Sigma, St. Louis, MO). The concentrations of the standard nucleotide solutions were calculated from optical density measurements using extinction coefficients (Voet et al., 1963). Fractions corresponding to deoxycytidine, deoxyadenine, thymidine and deoxyguanosine-5′-monophosphates were collected in 1–3 ml eluent in 20 ml scintillation vials. Scintillation cocktail (10 ml) was added and the radioactivity measured in a Packard Tricarb 1500 liquid scintillation counter for 20 min (Packard Instruments, Meriden, CT). Automatic quench correction was by an external standards method.

2.8. Fractionation of ^{3}H DNA by %G+C

DNA isolated from ^{3}H labelled soils was fractionated by ultra centrifugation on CsCl gradients containing bisbenzamide (Holben et al., 1993). Samples of ^{3}H-DNA (30 μg) were dissolved in CsCl solution (6 ml, 1.6 g ml^{-1}) containing bisbenzamide (0.2 μg ml^{-1}). The refractive index of the mixture was adjusted to 1.400, corresponding to a density of 1.707. Centrifugation to near isopycnic equilibrium was in a Sorvall TFT 45.6 ultra centrifuge rotor (Du Pont Instruments, Wilmington, DE) at 33 000 r.p.m. (110 000 *g*) for 96 h at 18°C. The density gradients were fractionated into 200 μl fractions by upward displacement with a dense immiscible fluid (Fluorinert, 1.87 g ml^{-1}, 3M, St Paul, MN) using an Isco model 640 gradient fractionator (Isco, Lincoln, NE). The DNA content of each fraction was estimated from OD measurements at 260 and 280 nm. The refractive index of a 40 μl sample of each fraction was measured in a refractometer. A 100 μl sample was digested with 400 μl 0.25M $HClO_4$ at 100°C for 30 min in a 20 ml scintillation vial, cooled to room temperature then mixed with 10 ml scintillation cocktail. The radioactivity of the digested ^{3}H DNA was measured for 20 min in a scintillation counter. Standard DNA mixtures (*Escherichia coli*, *Clostridium welchii* and *Micrococcus lysodeikticus*) of known %G+C, were used to calibrate the CsCl gradients so that %G+C could be derived from refractive index measurements.

2.9. Soil microbial biomass

Microbial biomass C was measured on six replicate 25 g samples of the test soils by the chloroform fumigation incubation method, using the procedure described by Horwath and Paul (1993).

2.10. Soil respiration in long-term incubations

Six replicate bulk samples of the soils were sieved to 5 mm and adjusted to a gravimetric water content of 19% which corresponded to a water potential of −300 kPa. Subsamples (150 g) of the soil were weighed into 1l glass canning jars. A 20 ml vial containing 2 ml 2M NaOH was included in the jars to trap CO_2. The closed jars were incubated in darkness at 25°C for 250 days. The base traps were replaced after 10, 20, 40, 80, 146, 180 and 250 days. The HCO_3^- content of the traps was determined by a dual end point titration with 0.1 M HCl from pH 8.3 to pH 3.7 (Horwath and Paul, 1993).

Cumulative CO_2 evolution curves were fitted to an exponential model of the form

$$C_{min} = C_1(1 - e^{-k_1 t}) + C_2(1 - e^{-k_2 t}) \quad (5)$$

Where C_{min} is cumulative mineralization, C_1 and C_2 are carbon pools and k_1 and k_2 the rate constants for the decay of these pools. A third compartment in the model (C_3) has a negligible decay rate on the time scale of this incubation. This has been established by carbon dating which has shown that about half the soil organic C is extremely resistant to decay with a half life of hundreds or thousands of years (Paul et al., 1993). We therefore constrained C_2 to equal half the total soil organic C minus C_1.that is

$$C_2 = C_{tot} - C_1 - C_3 \; and \; C_3 = C_1 + C_2$$

The parameters estimated from the curve fits

were used to calculate initial C mineralization rates for the soils:

$$\frac{\partial C}{\partial t} = C_1 K_1 + C_2 K_2 \quad (6)$$

The specific respiration rate (R) is equal to the sum of growth (G) and maintenance (m) related respiration rates. This was calculated as the initial mineralization rate divided by the microbial biomass (x)

$$R = G + m = \frac{\partial C}{\partial t}\frac{1}{x} \quad (7)$$

Maximum specific growth rates (v) can be estimated from specific respiration rates by assuming a growth yield efficiency (Y) of 0.6 and zero maintenance coefficient (m), from

$$vx = \left(\frac{\partial S}{\partial t} - mx\right)Y \text{ and } \frac{\partial S}{\partial t} \quad (8)$$

$$= Gx\frac{1}{(1-Y)} + mx \quad (9)$$

where S is microbial substrate combining Eqs. (8) and (9)

$$vx = Gx\frac{Y}{(1-Y)} \quad (10)$$

when m is zero, R=G for Y=0.6, x=1, v=1.33.R

3. Results

3.1. Respiration in soil slurries

Over short-term incubations (2 h) neither the formation of a slurry nor the addition of 750 nM thymidine affected the respiration rate of the soil. The rates of increase in headspace CO_2 concentration (Fig. 1) in the control, slurry and thymidine treatments were similar after the initial rapid increase on addition of water. The increase in headspace CO_2 concentration of about 1200 p.p.m. resulting from the formation of slurries was not a result of respiration as it also occurred in the formaldehyde treated soil. The increase

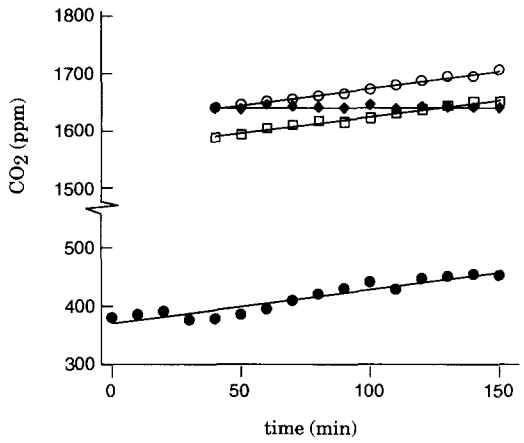

Fig. 1. Effect of slurry formation and thymidine addition on the respiration of soil at 15°C. Control soil incubated at 20% moisture without shaking (●–●). Slurry formed from 10 g soil plus 10 ml water, incubated with shaking at 60 r.p.m. (○–○). Slurry+750 nM thymidine (□–□). Slurry+0.1% formaldehyde (◇–◇).

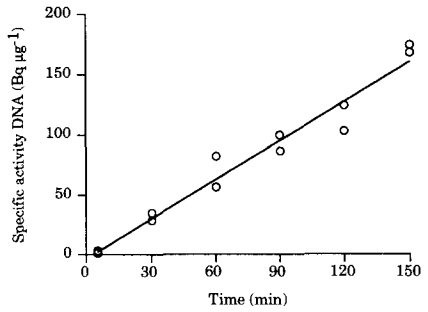

Fig. 2. The rate of ^3H incorporation into DNA of the soil microbial community of Corn–Soybean cropped soil, after addition of ^3H thymidine at 0.25 MBq nmol^{-1}.

was attributed to displacement of the soil atmosphere by the added water.

3.2. Time course of ^3H thymidine uptake

The specific activity of the DNA extracted from soil increased linearly for 150 min when incubated as a slurry with 0.403 μm ^3H thymidine (Fig. 2). The linearity of the thymidine uptake curve supports the evidence from the respiration measurements that the growth and activity of the soil bacteria did not change markedly over the course of the 150 min incubation.

3.3. Isotope dilution measurements

Plots of the effect of increasing additions of unlabelled thymidine (y+1) on the reciprocal of the specific activities of DNA extracted from the soils (1/x (1/Bq μg^{-1})) showed the expected linear form (Figs. 3 (a)–(c)). Dilution of the dTTP pool (p, Eq. 2), estimated by non-linear regression, varied from 0.07 to 0.14 nmol g^{-1} soil and was approximately 50% smaller in the reversion soil than in the Corn–Soybean treatment ($P<0.05$). The Grassland soil was intermediate and not significantly different from the other treatments (Table 1).

The estimates of the theoretical maximum specific activities of DNA in the absence of intrinsic isotope dilution (n, Eq. 2) and the replication rates (μ) derived from them using Eq. 3 are shown in Table 1. The DNA replication rate of the Reversion treatment was 50% higher than that of the Corn–Soybean rotation ($P<0.05$). The estimate of μ for the Grassland soil was in-

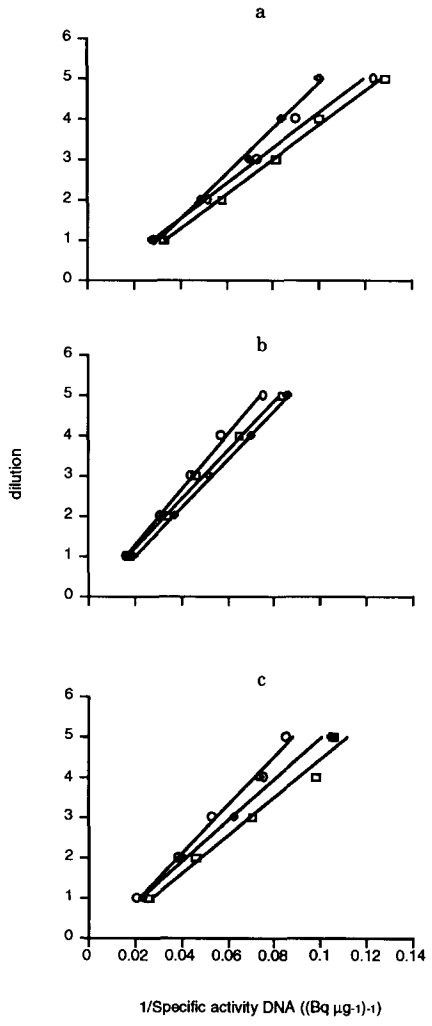

Fig. 3. Effect of isotope dilution on the incorporation of ^3H thymidine into DNA isolated from soils of three agronomic treatments (a) conventional Corn–Soybean rotation, (b) reversion to native successional community after conventional agriculture, (c) never ploughed hayfield. Different symbols (○, □, ◇) represent the means of duplicate samples of soils from three replicate treatment plots.

Table 1
Estimates of the maximum specific activity of ^3H DNA, intrinsic isotope dilution and specific replication rate in soils

Treatment	Specific activity DNA (bq μg^{-1})	±SD ($n=3$)	Intrinsic dilution (nmol Thy. g^{-1} soil)	±SD ($n=3$)	μ (h^{-1}) $\times 10^4$	±SD $\times 10^4$ ($n=3$)
Conventional	45.6	4.2	0.14	0.03	1.9	0.3
Reversion	64.7	5.4	0.07	0.01	2.7	0.4
Native	53.5	6.1	0.10	0.02	2.2	0.4

termediate and not significantly different from the other treatments (Table 1).

3.4. HPLC separation of nucleotides in ^3H DNA

The average base composition of DNA from the three soils was 68 mol% G+C, no treatment effect on this composition was detected. The dTMP fraction contained between 83 and 89% of the ^3H recovered in the nucleotide fractions (Table 2). The nucleotide fraction with the highest ^3H content after dTMP was deoxyguanosine 5′ monophosphate (dGMP), a pyrimidine which contained 5–10% of the total. It is probable that most of the ^3H found in the dGMP traction was due to incomplete chromatographic separation from dTMP rather than catabolism of ^3H thymidine. It would be expected that dispersal of ^3H from thymidine to other deoxynucleotides would lead primarily to ^3H deoxyadenosine 5′ monophosphate (dAMP), the other purine, by a salvage pathway.

3.5. Fractionation of ^3H DNA by %G+C

The %G+C and radioactivity profiles of the DNA from the conventional agronomic plots are shown in Fig. 4. Profiles of DNA from the other treatments were similar. The distribution of DNA of different base composition is typical of that of other aerobic soils (Holben et al., 1993), with a pronounced maximum at about 68% G+C. It is remarkable that the distribution of radioactivity closely mirrors that of total DNA. This means that actively replicating, thymidine incorporating bacteria were present in all %G+C fractions and that, on average, bacteria from all these fractions were replicating at the same rate.

3.6. Microbial biomass and specific respiration rates

Cumulative CO_2 evolution curves for the soils are shown in Fig. 5. The pool C_1 was 3.6 and 2.3 times larger in the Grassland and Reversion soils than in the Corn–Soybean rotation (Table 3). The size of this pool was the most important difference between the C mineralization curves for the three treatments. The rate constant k_1 was similar in the three soils.

The specific respiration rates were similar in the Corn– Soybean and Grassland treatments. The rate for the Reversion soil was about 50%

Table 2
Distribution of radioactivity in nucleotide monophosphates released by nuclease P1 digestion of ^3H thymidine labelled soil DNA

Specific activity (Bq $nmol^{-1}$)				
Nucleotide	Retention (min)	Corn/soybean	Reversion	Native
dCMP	2.8	2.3	3.0	2.5
dTMP	7.4	97.6	140.90	125.3
dGMP	10.9	5.1	4.2	4.1
dAMP	15.8	3.5	3.5	3.6

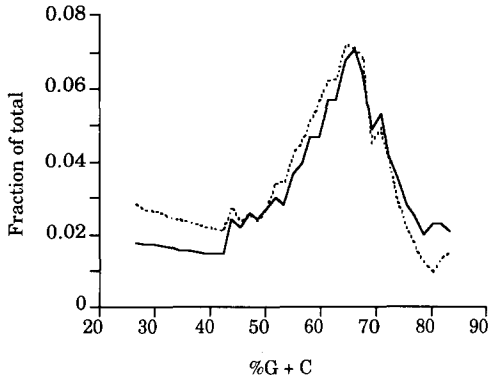

Fig. 4. Profile of ^3H labelled DNA from soil, fractions represent the proportion of total DNA (—) and radioactivity (----) at %G+C ranging from 20 to 80 mol%.

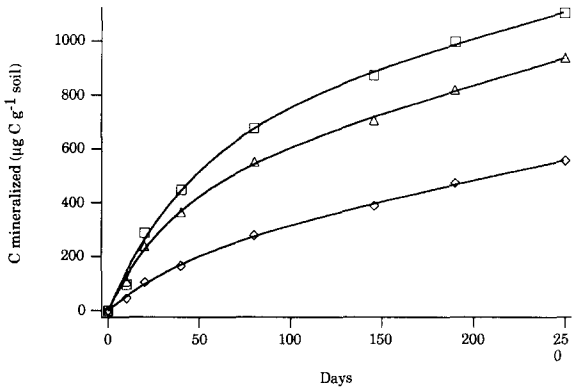

Fig. 5. Cumulative CO_2-C evolution from soils over 250 day incubation at 25°C. Corn–Soybean rotation (◇—◇), Reversion (△—△) and Grassland soils (□—□). Fitted curves are of the form $C_{min}=C_1(1-e^{-k_1t})+C_2(1-e^{-k_2t})$.

Table 3
First order rate constants and pool sizes fitted to long term (250 day) C mineralization from soils

Treatment	C_1 ($\mu g\,g^{-1}$)	k_1 ($days^{-1}$)	C_2 ($\mu g\,g^{-1}$)	k_2 ($days^{-1}$)
Corn/soybean	173 (24)	0.024 (0.005)	4327	0.00035 (0.00002)
Reversion	405 (32)	0.029 (0.003)	4095	0.00052 (0.00003)
Grassland	624 (102)	0.022 (0.005)	6876	0.00029 (0.00006)

Figures in parentheses are asymptotic standard errors of estimates.

higher than that of the Corn–Soybean treatment (Table 4). Maximum specific growth rates (v) estimated from these data (Table 4) are tenfold higher than the rates obtained from thymidine incorporation (Table 1). Corresponding minimum doubling times for the soil biomass are 8–11 days. The relationship between respiration rates of soils from different treatments was similar to that between thymidine incorporation rates; the Reversion treatment had the highest rate and the Corn– Soybean rotation the lowest. The effect of some other, more realistic, assump-

Table 4
Microbial biomass, specific respiration rates and stimated specific growth rates with some assumptions for growth yield and maintenance

	Treatment		
	Corn/soybean	Reversion	Grassland
Biomass C ($\mu g\ g^{-1}$ soil)	141 (14)	251 (17)	345 (26)
Soil respiration at $t=0$ (C_{min}) ($\mu g\ C\ g^{-1}\ h^{-1}$)	0.24 (0.02)	0.59 (0.03)	0.66 (0.06)
Specific respiration rate R ($\mu g\ C\ \mu g^{-1}$ biomass $C\ h^{-1} \times 10^4$)	16.8 (1.1)	23.3 (1.9)	18.9 (2.2)
Specific growth rate μ ($h^{-1} \times 10^4$)			
Assumptions[a]			
$Y=0.6, P_m=0$	25.2	35.0	28.4
$Y=0.6, P_m=0.8$	5.0	7.0	5.7
$Y=0.35, P_m=0.8$	1.8	2.5	2.0

Values in parenthesis are standard errors of the treatment means for biomass C and approximate standard errors of the estimates for respiration rates.
[a] Y, growth yield ($\partial x/\partial S$, when maintenance is zero). P_m, proportion of respiration derived from maintenance processes.

tions for Y and the proportion of respiration derived from maintenance process are shown in Table 4. If respiration-based growth rate estimates are to agree with those from thymidine incorporation, then either Y must be $\leqslant 0.35$, or $\geqslant 80\%$ of the substrate consumed by the soil biomass must be devoted to maintenance.

4. Discussion

The first problem in the measurement of microbial growth rates in soil is to assess the extent that the measurement procedures and their attendant disturbances affect the state and activity of the soil microbiota. Physical disturbance such as the production of soil slurries will change the chemical and physical environment of the soil bacteria and may thus affect their growth or metabolism. The measurements of respiration in slurried soils show no rate changes over the 2 h incubation and the rate of ^3H thymidine uptake remained constant for a similar period. These results suggest that the growth and activity of the soil microbial community did not change rapidly in response to the formation of slurries. Similar findings have been reported in a variety of studies on the metabolism of slurried or amended soils (Anderson and Domsch, 1978; Faegri et al., 1977; Bååth, 1990; Bååth, 1992).

If the soil microbial community can incorporate ^3H thymidine into its DNA at a rate which is characteristic of the rate of DNA synthesis in untreated soil, determination of this specific incorporation rate will give the specific rate of DNA synthesis in the soil. This can be treated as an absolute measurement of growth or could be related to biomass production by a factor equal to the average cellular DNA content. Most of the previous studies of ^3H thymidine uptake have been performed in aquatic systems with the intent of quickly measuring bacterial productivity. The extent of ^3H thymidine incorporation into DNA has usually been made by determining the amount of radioactivity present in a macromolecular fraction precipitated onto a filter (e.g. Moriarty and Pollard, 1981; Findlay et al., 1984; Thorn and Ventillo, 1988). There are problems with this approach which are exacerbated in the more complex matrix of soil. It is assumed that the radioactivity retained by the filter represents the total mass of thymidine in newly synthesized DNA extracted from the sample. There is no measurement of the mass of DNA so the specific activity cannot be determined directly. In aquatic systems with particulate organic matter, and es-

pecially in soils or sediments, the extraction of DNA is incomplete and an extraction efficiency for DNA must be determined (Thorn and Ventillo, 1988; Bååth, 1990; Bååth and Johansson, 1990). The estimate of thymidine incorporation is in mass units, the subsequent calculation of growth or replication rate requires either that the thymidine content of the standing biomass be estimated, usually from its DNA content (Fuhrman and Azam, 1982; Ellenbroek and Cappenberg, 1991) or by using an empirical thymidine conversion factor.

The combined uncertainties due to the use of extraction efficiency and thymidine conversion factors can be avoided if the rate calculations are based on the specific activities of the precursor and the product rather than on the mass of thymidine incorporated. In this study, by using recently available techniques for the extraction and purification of DNA from soil, we have been able to accurately determine the specific activity of the extracted DNA. Further, nuclease digestion of this DNA allowed us to measure the specific activity of the thymidine bases in the DNA and to demonstrate that the majority (85–90%) of the radioactivity in the DNA was present in this nucleotide. This finding demonstrates that the incorporation of ^{3}H into the DNA via catabolism of the added thymidine was minimal. Measurement of the specific activity of the thymidine bases in DNA, coupled with isotope dilution measurements of the specific activity of dTTP, allowed direct calculation of the DNA replication rate without the use of conversion factors which have caused so many problems.

The rate measurements are independent of the overall extraction efficiency of the DNA from the soil if the extracted DNA is representative of the whole soil microbial community. Bias could arise from the failure of the extraction technique to lyse some classes of organisms in the soil if the resistant organisms deviate from the mean rate of thymidine uptake. It is well known that bacteria in culture vary widely in their susceptibility to lysis by the hot detergent procedure used here and it is therefore probable that some of the soil bacteria were resistant to lysis. The mean yield of DNA ($10\ \mu g\ g^{-1}$ soil) was consistent with the complete recovery of DNA trom the total number of bacteria ($2.5\times10^{9}\ g^{-1}$ soil) if the average bacterial DNA content was 4 fg (c.f. Ellenbroek and Cappenberg, 1991). However, estimates of the extent of lysis based on DNA recovery are not strongly informative because of the uncertainty of the average DNA content of soil bacteria.

The DNA replication rates reported here will underestimate the true rate of replication to the extent that DNA from species which do not incorporate exogenous thymidine or DNA from dead organisms was included in the extract. In soils, the possible sources of 'non-incorporating' DNA which may be significant include fungi, extracellular DNA (presumably from dead organisms), and DNA from bacteria which do not incorporate thymidine into DNA. Other soil organisms such as protozoa and algae cannot significantly affect the soil or extract DNA content because their biomass and DNA content in most bulk soils is too low.

Bååth (1990) showed that an assemblage of 15 species of soil fungi failed to incorporate thymidine into DNA, therefore, unlabelled fungal DNA extracted from the soil would reduce the apparent replication rate. The DNA of the fungal hyphae in the soils used here represented less than 10% of the total DNA content, furthermore, this DNA was resistant to extraction by the hot detergent procedure used and was probably not present in the DNA extract (D. Harris, unpublished data, 1992). It is possible that DNA from fungal spores could have been included in the extract; we have no estimate of the extent of this contribution. Pre-extraction of the soil with buffer before cell lysis did not obtain measurable amounts of DNA suggesting that the contribution of extracellular DNA to the extract was negligible.

Thymidine uptake and incorporation into DNA are not universal in the bacteria and are absent or slight in eucaryotes (Pollard and Moriarty, 1984; Saito et al., 1985; Davis, 1989; Jeffrey and Paul 1990). The lack of thymidine kinase in bacteria appears to be the primary reason for the failure to incorporate thymidine. The species tested by Saito et al. (1985) included ex-

amples from important soil genera in which thymidine kinase activity was slight or absent. The proportion of soil bacteria which do not incorporate thymidine into DNA is unknown and is difficult to determine. Estimates could be made from tests on isolated organisms but we do not know how a set of cultured bacteria represents the majority of soil organisms.

Evidence trom the CsCl density gradient fractionation of ^{3}H-labelled DNA-bisbenzamide complex indicates that thymidine incorporation is widespread. The similarity of thymidine incorporation rates in all of the %G+C fractions may be a reflection of the enormous complexity of the soil microbial community (Torsvik et al., 1990). If each fraction contains DNA from many different species, and perhaps represents many physiological states of each species, then each %G+C fraction may be a large random sample with respect to the rate of thymidine incorporation. If so, then similar mean radioactivities between fractions would be expected.

Surveys of thymidine incorporation in hetertrophic bacteria (Saito et al., 1985; Davis, 1989; Jeffrey and Paul, 1990; Christensen, 1993) show that out of 190 isolates tested, 140 (74%) could be considered thymidine uptake positive. If this proportion is representative of the soil communities used here, the replication rates would be underestimated by a factor of 1.36 (1/0.74). This relationship means that the apparent replication rate is relatively insensitive to the proportion of bacteria which do not take up thymidine, until this proportion becomes large ($>> 0.5$).

The specific rate of respiration by the soil microbial biomass is related to the specific growth rate of the biomass by parameters representing growth yield efficiency (Y) and maintenance coefficient (m). Maintenance rates derived from chemostat cultures (Pirt, 1965; Chapman and Gray, 1981) are tenfold greater than conceivable average rates in soil (Gray and Williams, 1971; Shields et al., 1973). The assumption that the maintenance rate must equal the specific respiration rate for steady state populations (Anderson and Domsch, 1985) ignores growth and death in the soil population. Smith et al., (1986) assumed that maintenance rate and death rate were equal, based on simulations of C turnover in the soil biomass (McGill et al., 1981). However, the tracer dynamics which provided the observational basis of these simulations could be explained by maintenance processes alone, there is no necessity for growth (or death) to produce turnover in microbial C.

Simultaneous measurements of μ by tracer uptake into DNA and the specific respiration rate ($G+m$) on a number of soil samples in which the microbial biomass was growing at different rates could be used to estimate yield coefficient and maintenance rate. This follows from Eq. 10 which predicts a linear relationship between the specific growth rate and the specific respiration rate with a slope $(1-Y)/Y$ and an intercept of m. Estimates of Y and m from our data are not presented because the thymidine uptake and respiration measurements were not made in parallel on the same soil samples and only the three treatment means could be used for the regression.

The respiration based estimates of growth rate refer to the whole soil biomass and those based on thymidine only to the bacteria. This might be important if there were differential effects on the growth rates of fungi and bacteria in different treatments.

The maximal microbial biomass growth rates calculated from specific respiration rates (assuming $Y=0.6$ and $m=0$) are two to four times lower than growth rates estimated from thymidine uptake for sandy loam and acid organic soils by Bååth (1990, 1992). The measurements of growth rates by thymidine uptake reported here were less than 1/20 of those reported by Bååth. It seems unlikely that the true growth rates would differ so widely in soils and the disagreement between these results must be mostly due to differences in methodology. Other measurements of bacterial growth rates by thymidine uptake in soils have examined the rhizosphere (Christensen et al., 1989; Bååth and Johansson, 1992) where growth is expected to be more rapid than in bulk soils because of higher substrate concentrations. If the average growth rates in these soils were as high as those found by Bååth ($\mu=0.015$ h^{-1} at 25°C) or Clarholm and Rosswall (1980), minimum annual C inputs of 1200 to 3000 gC

m^{-2} $year^{-1}$ would be needed to satisfy the requirements for microbial substrate in the Corn–Soybean and Grassland soils (calculated from $-(\partial S)/(\partial t)=\mu x(1/Y)+mx$, for annual mean temp of 7 C, x=microbial biomass C, and assuming, $Q_{10}=2$, $Y=0.6$, $m,=0$). Such C requirements would greatly exceed the annual C input in these soils, which is less than 500 gC m^{-2} $year^{-1}$ (c.f. Swift et al., 1979; Buyanovsky et al., 1987). The soil respiration rates reported here would correspond to annual mineralization rates from the top 10 cm of soil of 190 gC m^{-2} $year^{-1}$ in the Corn–Soybean rotation soil, 450 gC m^{-2} $year^{-1}$ in the Reversion treatment and 510 gC m^{-2} $year^{-1}$ in the grassland soil if the same Q_{10} were assumed.

It is concluded that the probable average growth rates of the microbial communities in these soils were much closer to the rates predicted by thymidine uptake than to the maximum rates derived from mineralization measurements. The error in the thymidine uptake measurements due to the presence of DNA from non-incorporators was probably less than twofold. With this assumption microbial production is limited to 27 gC m^{-2} $year^{-1}$ in the Corn–Soybean rotation soil, 77 gC m^{-2} $year^{-1}$ in the Reversion treatment and 90 gC m^{-2} $year^{-1}$ in the Grassland soil. In a survey of freshwater and marine systems net primary productivity (NPP) averaged 258 g C m^{-2} $year^{-1}$ and bacterial production averaged 30% of NPP at 78 gC m^{-2} $year^{-1}$ (Cole et al., 1988), these averages for aquatic systems are similar to NPP for temperate terrestrial ecosystems and to the microbial production values estimated here. These estimates of microbial growth are much lower than those usually used in ecosystem models and would have important effects on production estimates of all higher trophic levels in the soil food web. It is important to realize that microbial production is not synonymous with the turnover of microbial C; this can also occur by maintenance processes, indeed this would appear to be the primary mechanism for C cycling in the microbial biomass of these soils.

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