

SHORT-TERM BACTERIAL GROWTH, NUTRIENT UPTAKE, AND ATP TURNOVER IN STERILIZED, INOCULATED AND C-AMENDED SOIL: THE INFLUENCE OF N AVAILABILITY

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Summary—Bacteria, *Pseudomonas paucimobilis*, were inoculated at two concentrations ($6.56 \times 10^4 \text{ g}^{-1}$ and $6.56 \times 10^9 \text{ g}^{-1}$) into sterilized soil amended with $700 \mu\text{g glucose-C g}^{-1}$. Two levels of $\text{NH}_4^+\text{-N}$ ($11.0 \mu\text{g g}^{-1}$ and $81.0 \mu\text{g g}^{-1}$) were used. The subsequent development was followed for three days by measurement of several biological, chemical and physiological parameters.

The amount of bacterial biomass-C ($\mu\text{g g}^{-1}$ soil) became twice as great in high as in low N treatments, and significantly decreased between 39.5 and 63.5 h for the high inoculum, high N level treatment due to decreasing cell size. By the end of the experiment the cumulative respired carbon was twice as great and more inorganic P was immobilized for high compared to low N treatments and all available $\text{NH}_4^+\text{-N}$ was taken up by the final sample time. Soil ATP concentrations were twice as large in high N treatments but the turnover times were twice as long compared to low N systems. The yield coefficient (Y), calculated from respiration and biomass-C values, equalled 0.61 while substrate was plentiful. Nitrogen limitation did not alter the efficiency with which glucose was transformed into biomass, but rather controlled the total amount of glucose used and biomass produced.

INTRODUCTION

Glucose is often added to soil to mimic the carbon inputs from roots. Peak production of CO_2 after glucose addition in preincubated natural soils (Chahal and Wagner, 1965; Oades and Wagner, 1971; Behera and Wagner, 1974) and sterilized, re-inoculated soils (Coleman *et al.*, 1978) usually occurs within the first 24 h and occurs before 40% of the substrate added is evolved as CO_2 . After 2–3 months of incubation, 20–30% of the added glucose-C remains in the soil (Shields *et al.*, 1973; Chahal and Wagner, 1965), the rest being lost as CO_2 . When glucose concentration is greater and the soil is not preincubated, peak rates occur later and are maintained longer (Nannipieri *et al.*, 1979).

Although microflora play an essential role in decomposition by producing hydrolytic enzymes, they compete with primary producers for uptake of mineralized nutrients (Hayman, 1975; Paul, 1976; Goring and Clark, 1948). This is especially likely to happen in the rhizosphere, where microfloral use of substrates such as simple sugars drives immobilization of inorganic nutrients (i.e. NO_3^- , NH_4^+ and PO_4^{3-}) which would otherwise be available for plants. Bacterial numbers alone are not sufficient for biomass determinations when bacterial sizes vary, especially under

conditions of rapid growth (Clarholm and Rosswall, 1980). Biovolumes, as determined from size classes, must be converted to dry weights or carbon values (Jenkinson *et al.*, 1976; Clarholm and Rosswall, 1980).

This experiment was designed to address several questions which arose during an earlier experiment (Anderson *et al.*, 1978; Coleman *et al.*, 1978; Cole *et al.*, 1978), and model simulations (C. V. Cole and H. W. Hunt, personal communication). We found some apparent discrepancies between model and experimental results in growth rates of bacteria in microcosms with sterilized and inoculated soil in the first 3 days. A subsequent comparison of the experimental results given here and various models for the growth of bacteria inoculated into sterilized soil are presented by Hunt and Cole (1983).

Our questions were: (1) Does CO_2 evolution reflect bacterial biomass formation in the first 1–2 days with an abundance of labile substrate (glucose, cellular metabolites) present? (2) Is there any uncoupled respiration (i.e. drastic change in Y , the yield coefficient) especially when cells are under nitrogen limitation? (3) Are growth rates the same at different inoculation levels? (4) Does nitrogen limitation significantly affect cell size, respiration, growth rates or N and P uptake? (5) Do ATP turnover times help us understand bacterial growth?

MATERIALS AND METHODS

The bacterial species used in this study (*Pseudomonas paucimobilis*) was isolated from the rhizosphere of *Bouteloua gracilis* [H.B.K.] Griffiths (An-

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derson *et al.*, 1978) and has been used extensively for other microcosm experiments (previously described as *P. cepacia*). The soil used was from the A horizon of the Renohill-Shingle complex (Renohill, a fine, montmorillonitic, ustollic haplargid, in intimate association with Shingle, a loamy, mixed, calcareous, mesic, shallow, ustic torriorthent) in a shortgrass prairie in northeastern Colorado. It is a fine sandy loam with ~15% soil moisture at field capacity.

Twenty gram quantities of soil were propylene oxide sterilized inside cotton plugged 50 ml conical flasks (Anderson *et al.*, 1978). The soil contained 11.0 $\mu\text{g NH}_4^+\text{-N g}^{-1}$ soil and 19.1 $\mu\text{g bicarbonate-extractable-P g}^{-1}$ soil after sterilization.

There were two inoculation levels, two nitrogen levels, three main sampling dates and three replicates per treatment. Glucose (700 $\mu\text{g C g}^{-1}$ soil) was added in 3 ml H_2O along with bacterial inocula of 0.065×10^6 bacteria g^{-1} or 6.56×10^6 bacteria g^{-1} . Bacteria were actively growing in exponential phase in RSSB medium mineral salts plus glucose, (Herzberg *et al.*, 1978) when inoculated. Nitrogen (70 $\mu\text{g N g}^{-1}$ soil as $(\text{NH}_4)_2\text{SO}_4$) was included in the inoculum to the high N treatments. Microcosms were sealed in 0.51 jars and incubated at 25°C. A 2-h sampling was taken for both inoculation levels at the high N level to determine initial values for bacteria (to determine death after inoculation), $\text{NH}_4^+\text{-N}$, $\text{PO}_4^{3-}\text{-P}$ and ATP. Subsequent samples (entire microcosms) were taken 15.5, 39.5 and 63.5 h after inoculation for a total of 42 microcosms. Initial $\text{NH}_4^+\text{-N}$ and $\text{PO}_4^{3-}\text{-P}$ were determined on sterilized but non-inoculated controls.

The following analyses were made on each microcosm: $\text{CO}_2\text{-C}$ evolution (Coleman *et al.*, 1978), inorganic phosphorus (Olsen *et al.*, 1954), $\text{NH}_4^+\text{-N}$ (Stanford *et al.*, 1973), and bacterial plate counts (Anderson *et al.*, 1978). The latter analysis was used for biomass estimates. Fluorescein isothiocyanate (FITC) stained slides, prepared according to van Veen and Paul (1979) were used for biovolume determinations by photographing random fields on each of two replicate smears with a Zeiss microscope at 2000 \times with an automatic compensating shutter so that the length of exposure varied and each picture obtained the same amount of light independent of the strength of the epifluorescence. The negatives were projected onto a screen where length and width determinations were made for each bacterium and calibrated to a photograph of a stage micrometer. Bacteria were considered cylinders with hemispherical ends for biovolume calculations. A total of 1255 cells were measured, with a range of 7–69 measurements per treatment replicate. Direct measurements of bacteria were also made on three samples using a filar micrometer.

The applicability to soil studies of the often used values of 20% dry weight and specific gravity of 1.1 determined for organisms grown in liquid culture has been questioned (van Veen and Paul, 1979). They recommended values of 50% dry weight and a specific gravity of 1.3 based on studies of bacterial growth over a range of moisture stress conditions. We used these latter values for conversion from wet weight and biovolume to dry weight, with bacterial-carbon calculated as 50% of dry weight. ATP was measured in bicarbonate extracts using a modification of the

method of Paul and Johnson (1977) as described in Fairbanks *et al.* (1983).

Two methods, based on different assumptions, exist for estimating yield coefficients. Determination of Y using ^{14}C -labelled substrates assumes all the substrate is used, and includes metabolite production as biomass. Alternatively, yield coefficients may be determined by direct measurement of microbial biomass (sizes and numbers), and the assumption that substrate used is the sum of biomass-C plus respired-C, as in the present experiment. The yield coefficient, Y , is defined here as the amount of biomass-C produced per unit substrate-C used exclusive of extracellular metabolites produced (which we did not measure). This value was calculated as

$$Y = \text{Biomass-C} / (\text{Biomass-C} + \text{Cumulative CO}_2\text{-C}).$$

We calculated ATP turnover rates based on respiration rates and ATP pool sizes, assuming 90.1 ng ATP produced per $\mu\text{g CO}_2\text{-C}$ respired with glucose as a substrate. This assumes that the pool size is constant over the time period during which the average respiration rate was calculated.

The primary data were subjected to analysis of variance to determine significance of treatments and treatment interactions. For the calculated data, values were calculated by treatment replicate and analysis run on these data. The highest level of significant treatment effects (ANOVA) were used to determine the means that were to be statistically compared and displayed in tables and figures. Tukey's honest significant difference value (Sokal and Rohlf, 1969) was applied as the mean separation test. We considered differences at the $P \leq 0.05$ level as significant.

RESULTS

The cumulative soil respiration (Fig. 1) showed significant differences in growth responses to inoculation level and nitrogen amendment. Respiration of populations inoculated at 100-fold lower levels lagged by approximately 17–18 h. By 63.5 h cumulative respiration in the low inoculation treatment approached that of the high, when amended with nitrogen. When no N was added, no bacterial growth was observed after 15.5 h at the high inoculation level or after 39.5 h at the low level, but CO_2 evolution continued.

Plate counts made 2 h after inoculation were only 15–20% lower than the added levels, indicating little mortality due to inoculation into soil. FITC direct counts agreed well with plate counts until numbers exceeded $1.0 \times 10^9 \text{ g}^{-1}$ soil, when the plate counts were 2-fold greater than direct counts (high N, high inoculum middle and late dates, and high N, low inoculum late date).

Bacterial plate count numbers reached peak values sooner at high compared to low inoculation levels (Table I-I). After 15.5 h there were more bacteria in N amended compared to unamended treatments (Table I-II). A high value of 2×10^9 cells per gram soil were observed in the high inoculum, high N treatment at the end of the experiment. Cell numbers never declined during the short duration of this incubation, even in the low N, high inoculum treatment. The shortest generation time observed was

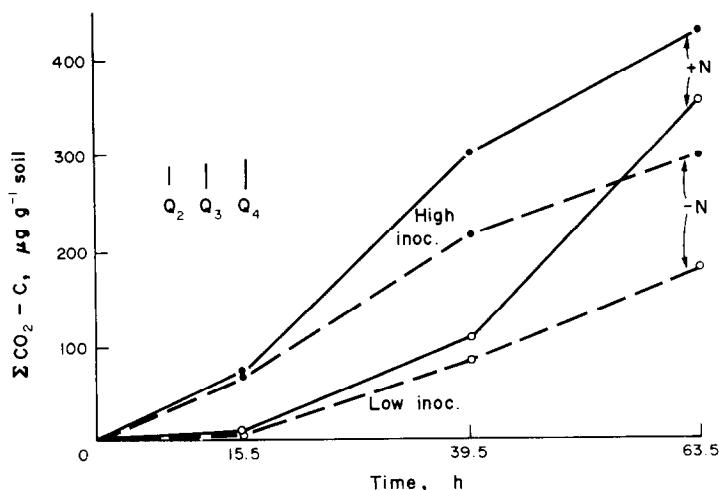


Fig. 1. The effect of N amendment and inoculation levels on cumulative $\text{CO}_2\text{-C}$ evolution by *Pseudomonas* in soil. Q values represent Tukey's HSD at the $P \leq 0.05$ level of significance.

2.4 h for the 0–15.5 h period low inoculum treatments.

Bacterial cell size decreased with time (Table 2). Cells at 63.5 h were only 0.8 as large as cells measured at 15.5 h. Averaging over time and N levels, cell sizes were significantly smaller for high inoculum treatments. The average size of bacteria was 1.16 times larger at high than at low N levels ($P \leq 0.06$).

Filar micrometer estimates were larger by 6% for the length and 50% for the width. The photographs

gave better resolution of the bacterial cell outline than was possible with direct visual observation.

Bacterial growth was more rapid in N-amended microcosms in both high and low inoculations (Fig. 2). The major increase in biomass carbon, for low inoculation treatments, was between times 1 and 2. At the higher inoculation levels, bacterial biomass increased 194-fold within the first 15.5 h but grew much more slowly after that. A doubling of bacterial biomass in response to N amendment occurred by 39.5 h. By 63.5 h at both inoculation levels, bacterial biomass in N-amended microcosms was double that in unamended microcosms. Nitrogen amendments did not have different effects on bacterial biomass production at the two inoculation levels, as indicated by the non-significant ($P = 0.944$) inoculation \times N interaction.

The best estimates of Y values for this experiment are from the early time (15.5 h) sampling for high inoculum (Table 3) and middle time (39.5 h) for low inoculum (Tables 3 and 4). These values are 0.65 and 0.59 (averaged over N levels) and are not significantly

Table 1. Bacterial plate counts ($\times 10^8$ per gram soil) as influenced by inoculation level by time interaction (I) and initial N level by time interaction (II)

BC I ($P < 0.001$)			
Inoculation level ($\times 10^6 \text{ g}^{-1}$)	Incubation time (h)		
	15.5	39.5	63.5
0.0656	0.428*	9.12	15.62
0.65	10.72	18.57	18.43

$Q_6 = 3.28$

$Q_3 = 2.65$

$Q_2 = 2.18$

II ($P = 0.001$)

Nitrogen level ($\mu\text{g g}^{-1}$)	Incubation time (h)		
	15.5	39.5	63.5
11.0	5.507*	12.05	12.22
81.0	5.64	15.63	21.83

$Q_6 = 3.28$

$Q_3 = 2.65$

$Q_2 = 2.18$

P values are probability values indicating the level at which the displayed levels of interaction are significant. Q_n values are values for which n numbers of means can be compared. Means differing by Q_n are significantly different at $P \leq 0.05$.

*Data presented as $\times 10^8$ cells g^{-1} soil.

Table 2. Bacterial biovolume per bacterial cell (μ^3) as influenced by inoculation level (I), nitrogen level (II), and time (III). P and Q values are explained in Table 1

I ($P = 0.0328$)		
	Inoculation level ($\times 10^6$)	
0.0656		6.56
0.407		0.343
II ($P = 0.057$)		
	Nitrogen level ($\mu\text{g N g}^{-1}$)	
11.0		81.0
0.344		0.400
III ($P = 0.0332$)		
	Time (h)	
	15.5	39.5
0.418		0.383
		63.5
		0.326

$Q_3 = 0.068$.

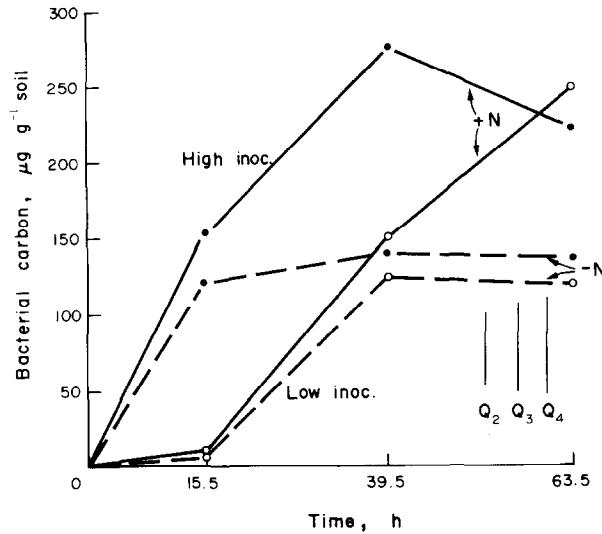


Fig. 2. The effect of N amendment and inoculation levels on bacterial biomass in soil incubations. See Fig. 1 for explanation of Q values.

Table 3. The effect of nitrogen amendment on bacterial growth responses after incubation at low inoculation levels (6.56×10^4 bacteria g^{-1} soil)

	Incubation time (h)					
	15.5		39.5		63.5	
	-N	+N	-N	+N	-N	+N
Substrate used, $\mu g C g^{-1}$ soil*	15	17	205	257	289	605
Biomass-C, $\mu g C g^{-1}$ soil	6	9	123	150	109	249
Yield	ND†	ND	0.60	0.58	0.38	0.41
Biomass-growth rate, h^{-1}	0.40	0.43	0.13	0.12	-0.005	0.021
NH_4^+ -N uptake, $\mu g N g^{-1}$ soil	1.8	ND	5.7	54.0	8.0	76.1
Apparent C:N	ND	ND	21.6	2.8	13.6	3.3
P uptake, $\mu g PO_4^{3-}$ -P g^{-1} soil	2.0	1.9	8.1	11.2	8.5	12.7
Apparent C:P	ND	ND	15.2	13.4	12.8	19.6
ATP, $\mu g g^{-1}$ soil	0.095	0.105	0.460	1.352	0.460	2.272
C:ATP	ND	ND	267	111	237	110
ATP turnover time, s	ND	ND	1.66	3.64	1.25	2.43

*Biomass-C + ΣCO_2 -C

†No determination because of lack of usable data at this early time period.

Table 4. Effect of nitrogen amendment on bacterial growth response after incubation at high inoculation levels (6.56×10^6 bacteria g^{-1} soil)

	Incubation time (h)					
	15.5		39.5		63.5	
	-N	+N	-N	+N	-N	+N
Substrate used, $\mu g C g^{-1}$ soil*	189	228	353	576	435	648
Biomass-C, $\mu g C g^{-1}$ soil	120	154	138	274	136	223
Yield	0.63	0.68	0.39	0.48	0.31	0.34
Biomass-growth rate, h^{-1}	0.33	0.34	0.006	0.024	0.00	-0.009
NH_4^+ -N uptake, $\mu g N g^{-1}$ soil	5.7	36.5	6.1	76.7	8.5	74.4
Apparent C:N	21.1	4.2	22.6	3.6	16.0	3.0
P uptake, $\mu g PO_4^{3-}$ -P g^{-1} soil	7.8	10.0	10.7	13.1	11.5	13.4
C:P	15.4	15.4	12.9	20.9	11.8	16.6
ATP, $\mu g g^{-1}$ soil	0.609	1.717	0.615	2.190	0.663	2.637
C:ATP	197	90	224	125	205	85
ATP turnover time, s	1.52	3.98	1.12	2.57	2.11	5.68

*Biomass-C + ΣCO_2 -C.

different at $P \leq 0.05$. N availability had an only slight influence (significant at $P = 0.06$) on Y .

92–94% of the KCl-extractable NH_4^+ -N in the N-amended treatments was taken up by 63.5 h at both inoculation levels (Tables 3 and 4). Apparent C/N ratios of bacteria ranged from 2.8 to 22.7 for high and low N treatments respectively.

There was a correspondence between timing of N and P uptake by the bacterial populations ($r = 0.72$, $P = 0.001$). Trends for available P uptake were similar to those for NH_4^+ -N uptake except a greater proportion of the total was taken up earlier. For example, 48% of the total immobilized N was taken up in the first period for the high N, high inoculum treatment while 74% of total immobilized P was taken up during this time. Bacterial C/P ratios, based on values of bacterial biomass-C and decreases in labile inorganic P, ranged from 11.8 to 20.9 (Tables 3 and 4).

Nitrogen addition effects on biomass-C and cumulative respiration were also reflected in higher ATP levels (Tables 2 and 3). However, C/ATP ratios were significantly lower in the nitrogen amendment, (104), vs the unamended (226), averaging over all times and inoculation levels. Turnover times of ATP were longer and pool sizes were larger for the high compared with low N treatments (Tables 2 and 3).

DISCUSSION

During the early stages of this experiment, bacterial growth was very rapid, with high yield efficiencies corresponding to rapid nutrient immobilization. Growth responses at the two inoculation levels were qualitatively similar, but the population at low inoculation level took *ca.* 18 h longer to reach levels of the population developed from the 100 × higher inoculation (Hunt and Cole, 1983). The amount of CO_2 evolved did indeed reflect bacterial growth rates, but only early in the incubation when substrate was readily available. There were only slight indications of lower growth efficiencies under nitrogen-stressed conditions.

Values for NH_4^+ -N uptake are underestimates of total N uptake since labile organic N compounds are released from the soil microbial population by sterilization procedures. The C/N values presented in Tables 2 and 3 are termed "apparent C/N ratios" because the amount of NH_4^+ removed from the soil solution is assumed to be the only N in the bacterial biomass. The ammonium N uptake underestimates the total N uptake, as there may be as much as $50 \mu\text{g g}^{-1}$ organic N present (Woods *et al.*, 1982), accounting for the unrealistically high apparent C/N ratios in the unamended treatments.

Luxury consumption and storage of P by bacteria is common [usually as polyphosphate (Harold, 1966)]. The relatively high bacterial P in this experiment (5–8%) is a reflection of the high extractable P content of this soil ($19 \mu\text{g PO}_4^{3-}\text{-P g}^{-1}$ dry soil).

Information on the yield coefficient (Y) is badly needed in development of budgets because it determines the C allocation and nutrient needs for bacterial production. There is considerable agreement on Y for glucose utilization under nonlimiting substrate

conditions, but Y has also been reported for conditions other than these (Payne and Wiebe, 1978). The theoretical maximum was shown to be 0.70 (i.e. 30% of C lost as CO_2) by Camp (1963). A pure culture population, growing aerobically without substrate limitations, yields a value of 0.60 (Payne, 1970). Shields *et al.* (1973) found 63% of added glucose-C still in the soil after 3 days with only traces of reducing sugars still present. Anderson *et al.* (1981) found 60% ^{14}C remaining for as long as nine days after single pulses of 100 or 600 μg uniformly-labelled glucose-C g^{-1} of soil using a single species of bacterium. They found Y values of 0.6 with bacteria alone by day 10 and 0.4 when nematodes grazed on the bacteria. This is consistent with the $Y \cong 0.35$ for mixed soil populations, which probably include grazers (Behera and Wagner, 1974; Waksman, 1929; Kaszubiak *et al.*, 1976; Sorensen and Paul, 1971).

The bacteria in our experiment were added from an exponentially growing culture into a nutrient rich, unpopulated soil environment, and bacterial growth was very rapid. The shortest generation time of 2.3 h was slightly longer than that reported by Behera and Wagner (1974) of 2.0 h, also in glucose amended soil.

Bacterial cell volumes observed ranged from 0.2 to $0.6 \mu\text{m}^3$. van Veen and Paul (1979) found sizes ranging from 0.46 to $0.87 \mu\text{m}^3$ for liquid and agar-grown cultures of soil bacteria. Time and N availability influenced sizes in our study. It is possible that in the high N treatments energy was limiting. Compared to direct counting the photographic method was considerably less tedious and time consuming for the dense populations encountered in our study.

Concomitant with the rapid increase in cell numbers, glucose was quickly utilized as evidenced by high respiration rates. We found $10 \mu\text{g CO}_2\text{-C g}^{-1} \text{h}^{-1}$ over the first 15.5 h at the high inoculation level (Fig. 3). Behera and Wagner (1974) reported $20 \mu\text{g CO}_2\text{-C g}^{-1} \text{h}^{-1}$ 10 h after glucose was added to pre-wet soil. However, they had larger initial bacterial levels ($5 \times 10^9 \text{g}^{-1}$) and their values were determined over a shorter time interval (5–10 h after substrate addition). Average values over several hours may not be indicative of actual peak rates due to the extremely dynamic nature of these populations.

The yield coefficient obtained for this experiment ($Y = 0.61$) is close to the expected value of $Y = 0.60$ for glucose utilization (Payne, 1970). The true values of Y , which are the substrate efficiency utilizations for biosynthesis, are those values where the substrate (glucose) is still in abundance. If all substrate was used, yet the cells continue to respire, then the obtained Y values are underestimates because the cumulative $\text{CO}_2\text{-C}$ values are inflated due to reutilization of material already synthesized. The principal hypothesis for this experiment, that bacteria would be less efficient at converting substrate to biomass under N limited conditions, was not supported.

Since we know that glucose is the dominant substrate used at the mid time period for the low inoculum and the early time period for high inoculum treatments, these comparisons most closely reflect the actual differences in ATP turnover rates between N levels.

The ATP concentration for the low N treatment

was one-half that of the high N treatment but the ATP turnover time was twice as long for the high N treatment. Thus ATP production was more similar than would be inferred from ATP concentrations. This is in conflict with trends noted by Karl (1980) for stressed, starved bacteria and warrants further investigation.

Under nitrogen stress, bacteria grew more slowly (Fig. 2) with no apparent change in efficiency of substrate utilization. For example, the bacteria in the high inoculum, high N treatment used 93% [(Cumulative CO₂-C + Biomass-C)/(glucose-C added) × 100] of the glucose by the last sample period but the low N treatment used only 62% and yet the apparent *Y* values were 0.34 and 0.31, respectively. Similarly, the high N, low inoculum treatment used 86% of the glucose by the last time period but only 41% for the low N treatment yet apparent *Y* values were 0.41 and 0.38, respectively. Lower yields at the final sampling time may represent a second population derived from cryptic growth upon the metabolites produced by the initial population (McGill *et al.*, 1973) or the metabolism of internal energy stores by the established population.

These results suggest that when N is limiting in labile-C rich areas in soil, the C substrate is not readily used but may be present for use as N becomes available. In situations where microbial grazers are present (i.e. bacteriophagic nematodes or protozoans) and N or P is mineralized by them (Coleman *et al.*, 1978; Cole *et al.*, 1978; Anderson *et al.*, 1981; Woods *et al.*, 1982), substrate-C utilization should be stimulated under inorganic nutrient-limited conditions, as predicted by the simulation model of Hunt *et al.* (1977).

In summary, we list answers to the questions posed earlier:

(1) Carbon dioxide evolution reflects bacterial biomass formation but only when abundant substrate is available.

(2) Respiration is not uncoupled (i.e. drastic changes in the yield coefficient) because of limitation of N.

(3) Inoculation level does not affect growth rates as long as the substrate is plentiful, even though the substrate remains abundant longer when the initial bacterial population is smaller.

(4) Nitrogen stress significantly affects aspects of bacterial cell size, respiration, growth rates and N and P uptake, but not the efficiency with which substrate is used to synthesize biomass.

(5) The turnover rate of bacterial ATP is greater but the pool size is smaller when N is limiting, resulting in generally similar total ATP production in populations growing in low and high N environments.

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