THE ROLE OF MICROORGANISMS IN THE METABOLISM OF ^{14}C-LABELED PLANT MATERIALS BY WOODLICE (*TRACHEONISCUS RATHKEI* BRANDT)

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THE ROLE OF MICROORGANISMS IN THE METABOLISM OF ^{14}C-LABELED PLANT MATERIALS BY WOODLICE (Tracheoniscus rathkei Brandt)

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ABSTRACT

THE ROLE OF MICROORGANISMS IN THE METABOLISM OF ^{14}C-LABELED PLANT MATERIALS BY WOODLICE (TRACHEONISCUS RATHKEI BRANDT)

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Uniformly labeled ^{14}C-cottonwood leaves and ^{14}C-wheat stems and leaves were fed to woodlice alone or in combination with soil microorganisms and with or without antibiotics. Degradation of the two plant materials was more rapid when the activities of soil animals and soil microorganisms were combined regardless of the presence or absence of antibiotics. A pulse feeding of antibiotics did not significantly affect the metabolism of the two plant materials over a long period but did affect respiration of ingested label one day after the pulse. Antibiotics did not affect the rate of metabolism of previously assimilated label. Decomposition was affected by the composition of the material. After 33 days, 60.3% of cottonwood carbon and 29.0% of the wheat carbon was respired by the animals, 35.3% and 64.1%, respectively, were excreted and 15.9% and 8.3%, respectively, were retained in the body. The rate of degradation of the faeces was slower than for the original plant material. Of the label

assimilated by the animals, 78.1% and 79.8%, respectively, were used for maintenance consumption. The final elimination rate was 0.7% and 0.6% of the assimilated label, respectively. Based on the antibiotic studies, microorganisms in the gut appear to play a minor but significant role in the metabolism of plant materials by woodlice.

The gut flora of woodlice was also studied to determine their role in the metabolism of organic materials by soil animals. Animals kept in the laboratory for 50 days maintained a rather constant population of about 18 X 10^4 microorganisms per gut. About 50% of this population was found to be facultative anaerobes; no obligate anaerobes were observed. Starvation or treatment with antibiotics drastically reduced this population suggesting organisms must be growing to maintain their population density against digestion and elimination. Bacteria that were dominant in the gut and faeces were not prevalent in the natural animal foods which suggests a resident gut flora. Two dominant members of the gut community were isolated and identified to be Pseudomonas and Flavobacterium. ^{14}C-labeled Flavobacterium cells fed to woodlice were extensively digested and the contents assimilated by the animal. Microorganisms appear to be growing and being digested in the gut at the same time.

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By

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A DISSERTATION

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To my wife, Zon, for the unceasing inspiration she provided to complete this work

To the creatures of the soil who work day and night year after year, yet do not ask anything in return except for our moral obligation to protect them

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INTRODUCTION

The cooperative role of soil animals and soil microorganisms in the decomposition of biological materials is gaining recognition because of the importance of both in processing the complex variety of materials entering our terrestrial ecosystem. Traditionally, only soil microorganisms have been studied extensively to explain the intricate nature of the biological process of decomposition. But now the soil animal, which might be considered analogous to the plow, has been considered important in the mixing of mineral and organic fractions of the soil. The low available energy content of their natural substrate makes them efficient in mobilizing and comminuting large amount of organic residues. Hence, in this study, the ability of the animal to digest microorganisms associated with the substrate could be reasonably believed to be related to the nature of the substrate. Substrates with low available energy content are not able to support microbial growth and consequently cells die, lyse and the contents assimilated. Those substrates high in available energy as in the case of newly fallen leaves are readily attacked by microorganisms presumably making them more palatable to the animal. The animal could then benefit by ingesting these foods higher in available nutrients.

Similarly, substrates that are naturally high in available nutrients could also be a suitable food for the animal. The subsequent contact of the faecal materials with the soil further hastens the degradation of organic matter in the soil. Thus, recent advances in pest control management now seriously take into consideration the possible harmful effects of chemicals on the biota of the soil.

Therefore, this study was conducted to further understand the relationships between soil animals and soil microorganisms in the degradation of organic matter in the soil.

Chapter I

METABOLISM OF ^{14}C-LABELED PLANT MATERIALS BY WOODLICE (*Tracheoniscus rathkei* Brandt) AND SOIL MICROORGANISMS

BACKGROUND

The feasibility of using ^{14}C-labeled substrates in studying the decomposition of organic materials by soil animals and soil microorganisms was previously demonstrated (Reyes and Tiedje, 1973). Although we used yeast cells as the substrate in that study, the results concurred with the findings of many soil biologists that soil animals and microorganisms play synergistic roles in degrading organic materials in the soil. Since the substrates under natural conditions are of plant origin, we conducted a similar study using ^{14}C-labeled cottonwood leaves and the aboveground portion of ^{14}C-uniformly labeled wheat. Experiments were conducted to determine 1) the effect of the combined degradative activity of soil animals and soil microorganisms and 2) the relative role of the microorganisms in the alimentary tract of isopods in digestion by using antibiotics.

MATERIALS AND METHODS

Soil animal

Tracheoniscus rathkei Brandt, a common woodlouse in the woodlands of this region was used in this study. They were collected from campus woodlots and kept in the laboratory as described in the previous study (Reyes and Tiedje, 1973). The animals were maintained on wood gathered from the collection site.

Substrate

Cottonwood leaves (*Populus deltoides*) from seedlings labeled at their 16-leaf stage (Larson, Isebrands and Dickson, 1972) were kindly furnished by Forest Service, USDA, Rhinelander, Wisconsin. Wheat (*Triticum sativum*) grown to 170 days was obtained from the Federal Experiment Station for Agricultural Chemistry, Vienna, Austria. Chemical fractionation analyses performed on similarly grown wheat showed that lignin and acid hydrolyzable fractions composed 42% (Zeller, Oberländer and Roth, 1968), suggesting that large amounts of label are in resistant forms. The uniformity of the labeled plant materials was further assured by grinding the dried tissue in a Wiley intermediate mill (A. H. Thomas Co., Phila., Pa.) with 40-mesh delivery tube. They were stored in screw cap bottles inside a desiccating jar containing silica gel. The specific activity of ground cottonwood leaves was 0.0061 μC/mg and the wheat was 0.0512 μC/mg

dry weight.

Non-labeled cottonwood leaves and wheat were prepared in the same manner as the labeled plant materials. Sterile distilled water was used whenever water was needed. An aqueous antibiotic mixture of 500 ppm chlortetracycline and tetracycline (ICN Nutritional Biochemicals Corp., Cleveland, Ohio) which was previously shown to reduce the bacterial population of the gut (Reyes and Tiedje, 1974b) was also used in this experiment.

Metabolism experiment

Methods similar to those used in the previous study (Reyes and Tiedje, 1973) were used to contain animals, trap $^{14}CO_2$, and measure the amount of radioactivity in the samples.

The experiment was laid out as 2^3 factorial in completely randomized design with plant materials, soils, and antibiotics as the factors each at two levels with four replications. Three isopods were placed in a jar containing either charcoal-plaster of Paris or 15 g of freshly collected soil containing the natural microbial population. The animals in the jar were fed with 2.5 mg of either cottonwood or wheat placed on a glass cover slip. The labeled food was moistened with 10 µl of either water or antibiotic mixture. Non-labeled cottonwood or wheat was fed to the animals 1 day after the initial feeding of the label and at every inspection thereafter. Inspections were made after 1-3, 5-7, 9, 12, 15, 19, 23, 27, 32, and 33 days; after each inspection,

trapped $^{14}CO_2$ was determined. Faeces in jars with charcoal-plaster of Paris were composited and the radioactivity determined after 1, 2, 5, 19, and 23 days. The faeces in jars with soil were not removed to allow metabolism by soil microorganisms. The animals were collected at the end of the experiment, dried at 60°C and stored in the desiccating jar prior to radioactivity determination.

To ascertain the effect of the antibiotics directly on the animals, three isopods per jar on charcoal-plaster of Paris were allowed to consume 2.5 mg of labeled cottonwood and the respired $^{14}CO_2$ was measured after 1 day. After this sampling period one-half of the animals were fed with non-labeled cottonwood moistened with water and the other half were fed cottonwood but moistened with the same antibiotic mixture used in the preceeding experiment. The eliminated $^{14}CO_2$ was measured every day for 2 more days. The reported values are averages from four replicates.

The mineralization rate in the soil of labeled plant material and faeces from animals that either did or did not receive antibiotics was compared. Several isopods were fed with labeled cottonwood leaves moistened with either antibiotic mixture or water. All of the faeces for 2 days were collected, dried, weighed and divided. Each of these faecal materials and 2.5 mg of labeled cottonwood was spread on the surface of freshly collected soil in a jar. Respired $^{14}CO_2$ was measured daily for the first 6 days and then every other day until 16 days. The treatments of labeled cottonwood and

faeces from untreated animals were replicated three times but the treatment of faeces from antibiotic treated animals were replicated twice due to limited amounts of faeces. The faeces from antibiotic treated and untreated animals had 2.2×10^{-4} μC/mg and 1.3×10^{-4} μC/mg, respectively.

All of the values were transformed by arcsin (angular) transformation before being subjected to statistical analysis (Sokal and Rohlf, 1969).

RESULTS

Plant material metabolism

The combined activities of soil animals and soil microorganisms were significantly more effective in degrading either cottonwood or wheat (Table 1) with 83.7% and 61.4% of the plant carbon converted to CO_2, respectively. In the absence of soil microorganisms, only 60.3% and 29.0% were respired, respectively. The amount of label recovered in the faeces from animals in jars without soil and antibiotic treatment was 35.3% and 64.1% for cottonwood and wheat diets, respectively. The higher respiration and assimilation and the lower excretion values show that cottonwood was more readily degraded by the animals than wheat.

Figure 1 shows the progressive metabolism of cottonwood and wheat, respectively, with or without soil. Clearly, the presence of both soil animals and soil microorganisms stimulated decay over the animals alone. Also, the

Table 1. Fate of uniformly labeled ^{14}C-cottonwood and ^{14}C-wheat 33 days after feeding to woodlice.

Treatment	Fate of the label (% of original label)			
	Respiration	Faeces	Animal	Recovered Label
Cottonwood				
No antibiotic				
No soil	60.3±0.83#	35.3	15.9±1.32@	111.5
With soil	83.7±0.83	N.D.	11.5±1.32	95.2
	72.0±0.59		13.7±0.93	
With antibiotic				
No soil	61.9±0.83#	34.3	14.2±1.32@	110.4
With soil	86.0±0.83	N.D.	10.8±1.32	96.8
	73.9±0.59 73.0±0.41*		12.5±0.93 13.1±0.66*	
Wheat				
No antibiotic				
No soil	29.0±0.83#	64.1	8.3±1.32@	101.4
With soil	61.4±0.83	N.D.	4.8±1.32	66.2
	45.2±0.59		6.6±0.93	
With antibiotic				
No soil	27.7±0.83#	62.8	8.6±1.32@	99.1
With soil	59.7±0.83	N.D.	6.6±1.32	66.3
	43.7±0.59 44.5±0.41		7.6±0.93 7.1±0.66	

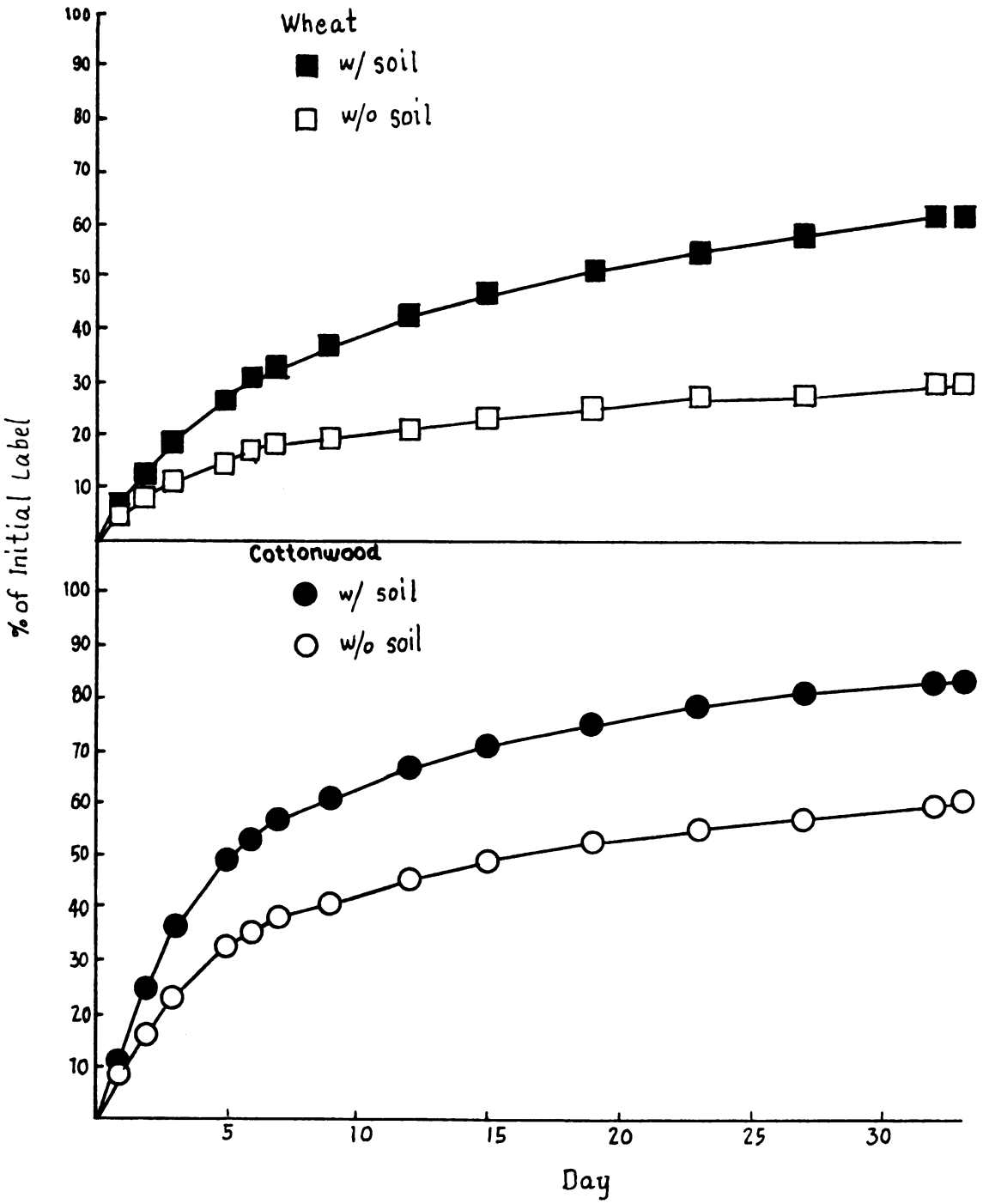
N.D. - Not determined

*Different from wheat ($P < 0.001$)

#Different from soil ($P < 0.001$)

@Different from soil ($P < 0.01$)

Figure 1. Cumulative production of $^{14}CO_2$ from ^{14}C-labeled cottonwood and wheat in treatments with and without soil. Note - only treatment without antibiotics was plotted since antibiotics did not have a long-term effect on the respiration.


Wheat
w/ soil
w/o soil
Cottonwood
w/ soil
w/o soil
% of Initial Label
Day
100
90
80
70
60
50
40
30
20
10
5
10
15
20
25
30

combination of both groups was superior to soil microorganisms alone since the 16-day mineralization value for cottonwood was 72% (Figure 1) compared to 48% (recalculated from Figure 3) for the microorganisms alone. Also from Figure 1 it can be seen that the presence of soil stimulated metabolism on the first and second days possibly due to direct microbial colonization of the substrate. This could be the explanation for the significantly lower amount of label assimilated by the animal in the presence of soil (Table 1).

The inclusion of antibiotics in the diet during the initial feeding of the labeled plant materials did not significantly affect the degradation of plant materials either in the presence or absence of soil over the 33-day period. However, antibiotics had a measurable effect during the first day of the feeding experiment when their effect should have been maximum (Table 2). The antibiotic effect on respiration disappeared by the second day. The antibiotics, however, had no effect on the animal's metabolism of previously assimilated label (Table 2) suggesting that the antibiotic effect was not on the animal but on microorganisms within the gut.

The presence of antibiotics in the initial diet also had no effect on the values for label recovered in the faeces (34.3% and 62.8%, respectively). Although these faecal values were from composited samples, a finding similar to that in the previous analysis of variance could be inferred where antibiotic was shown to have no long-term

Table 2. Effect of antibiotics on animal respiration of ingested ^{14}C-labeled substrates, and on respiration of ^{14}C-assimilated label.

Source of label	Incubation time (days)	Cottonwood		Wheat	
		Antibiotics	No antibiotics	Antibiotics	No antibiotics
		% of original label			
Ingested[#] food	1	5.6*	8.2	2.7*	4.7
	2	8.5	7.8	3.6	3.7
	3	6.6	6.9	2.9	2.6
Previously[##] assimilated	0	8.5±1.8	8.2±1.5	--	--
	1	7.3±1.8	7.2±0.7	--	--
	2	4.9±0.4	5.7±0.8	--	--

[#]Standard error is 1.33 for each mean

[##]Standard error is less than 1.78 for each mean

*Difference between antibiotic and no antibiotic is significant at $0.05 < P < 0.10$; other differences are not significant.

effect on the metabolism of the plant materials by the isopods. The cumulative loss of label through the faeces is depicted in Figure 2. The difference in the biodegradability of the cottonwood and the wheat by the isopods is also apparent from the rate of label defaecation. As in total respiration, the nature of the food significantly affected the label absorbed by the animals. The animals assimilated cottonwood better than wheat; and based on representative values, 15.9% and 8.3%, respectively was in stable body components at the termination of the experiment.

The bulk of the label appeared to have been excreted 2 days after ingestion of the label (Table 3). During that period, the animals that ate cottonwood discharged 39.3% of the initial label while those fed wheat excreted 60.2%. The remaining label, composed of animal residual value and the respective total for CO_2 and faeces, is presumed to have been assimilated by the animals as in the case of the previous study (Reyes and Tiedje, 1973). During the next 31 days, most of the assimilated label was respired instead of being excreted (61.5% and 16.6% for cottonwood and 49.9% and 29.9% for wheat, respectively). Thus, maintenance energy was calculated to be 78.1% and 79.8% for cottonwood and wheat fed animals, respectively.

Although the elimination rate of the assimilated label was different over time depending on substrate (Table 3), the total amount that was excreted was virtually identical (12.0% and 12.3%). The final elimination rates of 0.7% and

Figure 2. Cumulative elimination of label through defaecation.

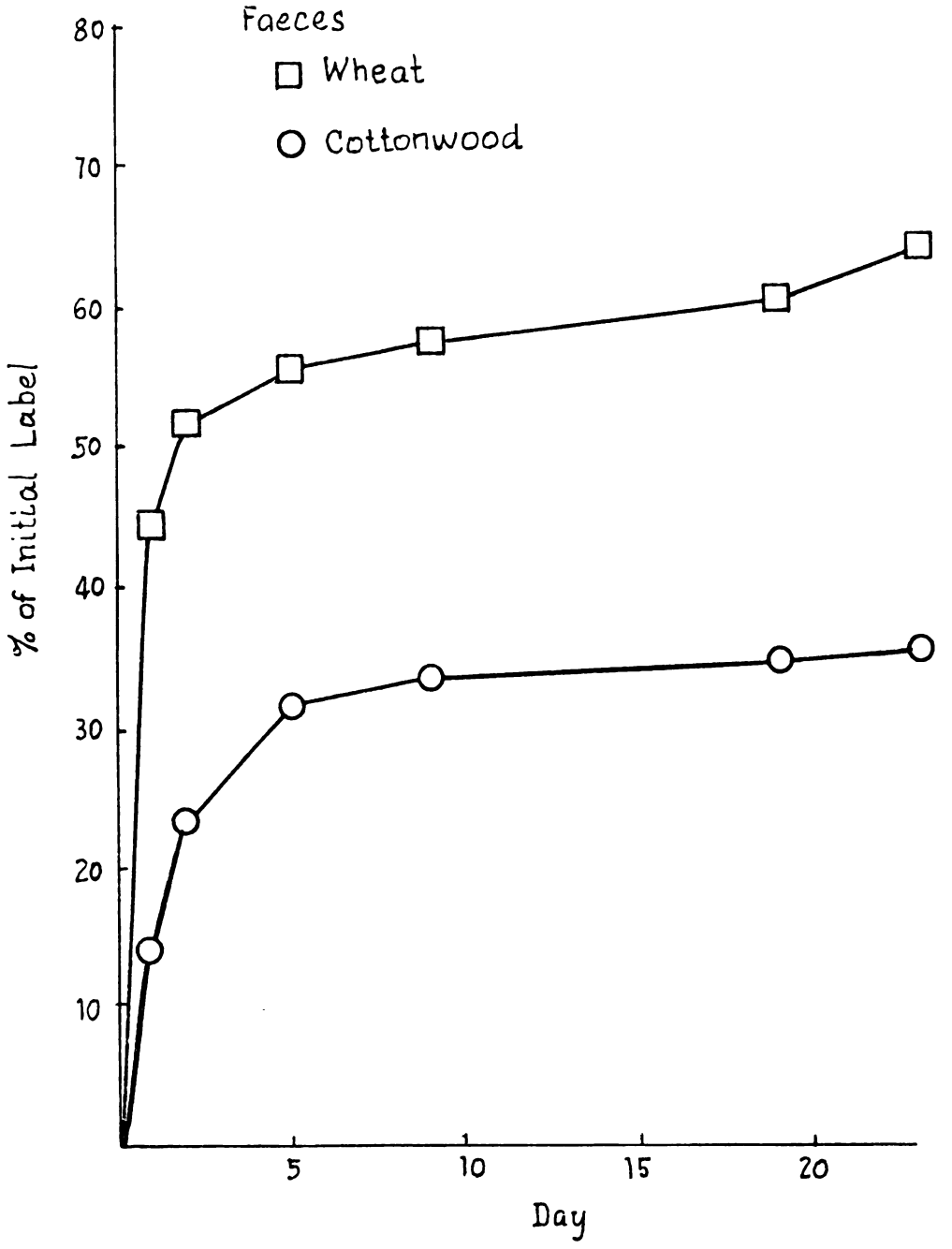

Faeces
Wheat
Cottonwood
80
70
60
50
40
30
20
10
% of Initial Label
5
10
15
20
Day

Table 3. Progressive loss of ^{14}C-label in cottonwood and wheat from the animals as CO_2 and faeces.

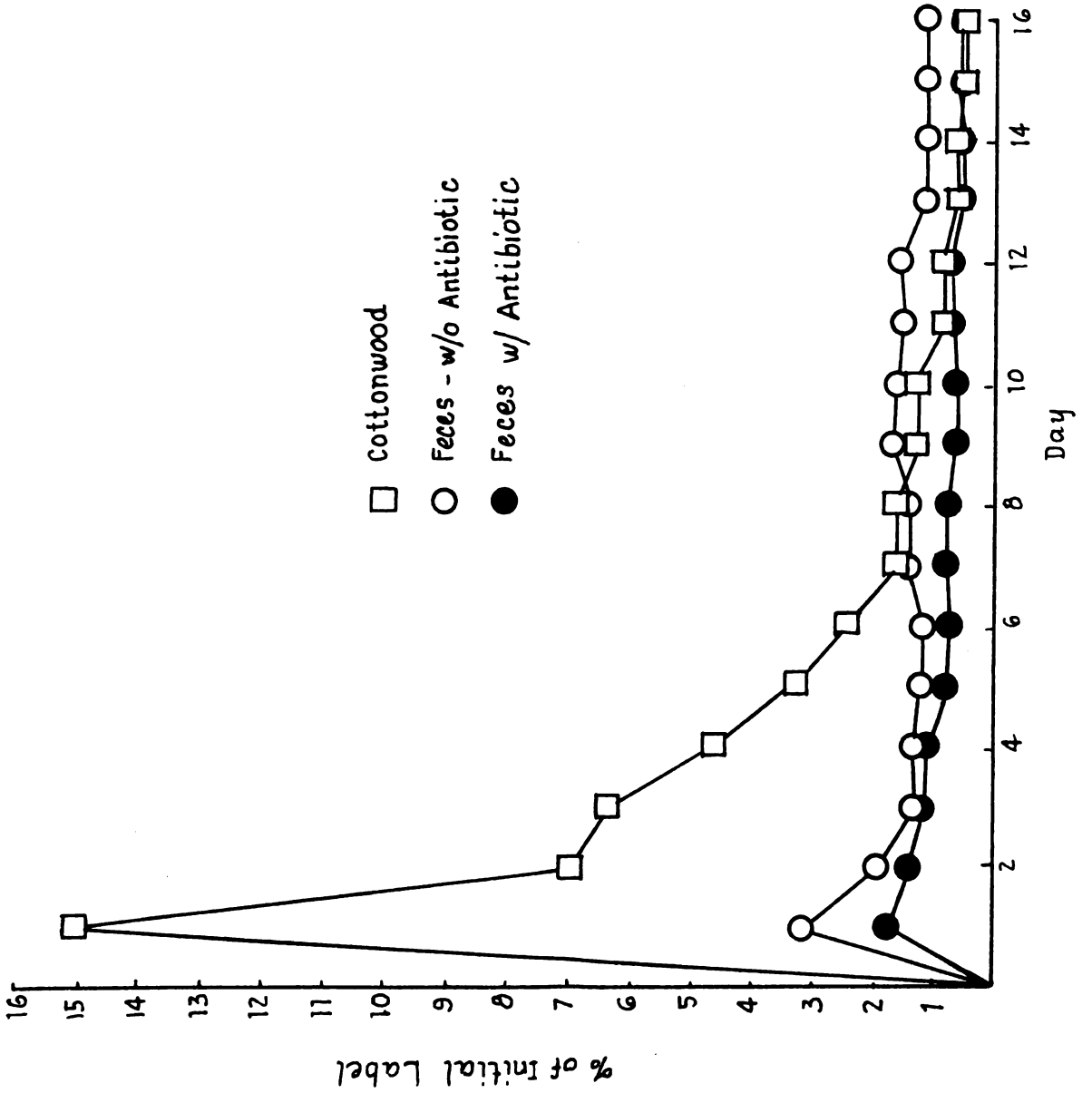
Time period (days)	Percent of original label lost in time period			Percent of assimilated label lost (cumulative)			Elimination rate; % assimilated label/day#		
	CO_2	Faeces	Total	CO_2	Faeces	Combined	CO_2	Faeces	Combined
				Cottonwood					
0-2	(15.9)*	(23.4)	(39.3)	--	--	--	--	--	--
3-9	25.8	10.2	36.0	35.7	14.1	49.8	5.1	2.0	7.1
10-19	10.9	1.1	12.0	50.8	15.6	66.4	1.5	0.2	1.7
20-23	2.8	0.7	3.5	54.7	16.6	71.3	1.0	0.3	1.3
24-33	4.9	N.D.	4.9	61.5	N.D.	78.1	0.7	N.D.	0.7
Residual			15.9			22.0			
Total	44.4	12.0							
				Wheat					
0-2	(8.4)	(51.8)	(60.2)	--	--	--	--	--	--
3-9	11.3	5.8	17.1	27.4	14.1	41.5	3.9	2.0	5.9
10-19	5.5	2.7	8.2	40.7	20.7	61.4	1.3	2.1	3.4
20-23	1.4	3.8	5.2	44.1	29.9	74.0	0.9	2.3	3.2
24-33	2.4	N.D.	2.4	49.9	N.D.	79.8	0.6	N.D.	0.6
Residual			8.3			20.1			
Total	20.6	12.3							

*Values in parenthesis are not included in the calculation of elimination rate

#Elimination rate values are averages for the indicated time period

N.D. - Not determined

Figure 3. Percent respiration per day of cottonwood leaves and faeces added to soil.


% of Initial Label
Day
Cottonwood
Feces - w/o Antibiotic
Feces w/ Antibiotic

0.6%, were also similar. This suggests that after the animals have utilized the digestible substances from their food, the fate of the label was the same, being governed only by the inherent metabolic pathways of the animal.

Biodegradability of cottonwood and faeces

The biodegradability of the faeces collected 2 days after feeding labeled cottonwood to the animals and later introduced into the soil was affected by the presence of antibiotics in the diet. After 16 days, 23.7% of the label present in the faeces from non-treated animals was metabolized by soil microorganisms compared to only 13.5% of the faeces from antibiotic treated animals.

The daily respiration rate in the soil (Figure 3) shows that cottonwood which has not passed the gut was better degraded than faeces, supporting the explanation that the animal is utilizing the readily available compounds.

DISCUSSION

The result of this study using ^{14}C-uniformly labeled plant materials agrees with the earlier finding using yeast cells that the concerted action of soil animals and soil microorganisms resulted in a more rapid and complete degradation of organic materials (Reyes and Tiedje, 1973). This synergistic relationship is very important particularly in forest ecosystems receiving large amounts of surface litter annually. The importance of this relationship was

emphasized by Mills and Alley (1973).

As found in our previous study, the high rate of decomposition of plant materials was primarily due to the metabolism by the animals and the subsequent decomposition in the soil of the unassimilated labels associated with the faeces.

The microorganisms in the alimentary tract of the animals seemed to have had a significant effect on decomposition of the plant residues as evidenced by the effectiveness of the antibiotics in reducing the $^{14}CO_2$ respired during the 1-day period when they were introduced into the gut together with the labeled material. This suggests that microorganisms in the gut might have a dual role in the decomposition process mediated by soil animals because of the observation that microorganisms have nutritional value to the animals (Reyes and Tiedje, 1974a).

Considering that natural materials are poor in nitrogen and other substances except carbon, it is reasonable to believe that bacteria and fungi containing their own catabolic enzymes grow on these food sources and later become a source of other essential nutrients for the grazing animals. Danilewicz (1972) found that Pseudomonas isolated from insect larvae growing on Populus "Hybrida 277" were able to degrade lignin and to a certain extent pectin but not cellulose.

It is well documented now that isopods also produce digestive enzymes (Schmitz and Schultz, 1969; Clifford and Witkus, 1971; Donadey, 1972; Donadey and Besse, 1972).

Among these enzymes are proteases (Hartenstein, 1964) cellulase, chitinase, and lipase (Hartenstein, 1970) secreted from the hepatopancreas. This organ also acts as an absorbing and storage facility.

The metabolism of nitrogenous substances contributes to energy production of terrestrial isopods (Weiser, 1972). As much as 107.0 μg NH_3-N/d-day in Porcellio scaber is produced with a corresponding consumption of 4.33 ml 0_2/g-day. Arginine, essential to isopods for protein and phosphagen (arginine phosphate) biosynthesis (Hartenstein, 1970), could come only from protein and free amino acids in microorganisms. From this nutritional standpoint, the microorganisms enrich the animal foods either by nitrogen fixation (Jurgensen and Davey, 1971; Seidler et. al., 1972; Sharp and Millbank, 1973) or the concentrating effect of microbial cells in the substrates (Knutson, 1972). Anderson (1973) found that nitrogen accumulated in woodland leaf litter and suggested that nitrogen came from a large volume of wood and bacteria being recycled in the habitat with fungal mycelia and fruiting bodies acting as sink for nutrients against leaching (Stark, 1972). Stark (1972) also found that fungal rhizomorphs are excellent sources of Ca, Cu, Fe, K, Mg and Mn besides N and P. Thus, where microorganisms cannot actively metabolize organic substrates in the animal gut, they can be very important in supporting the activity of soil animals.

The difference in biodegradability of cottonwood and wheat was most likely due to the difference in biochemical

composition of the two plant materials. Though the specific activity of the components of a uniformly labeled plant is the same (Jenkinson, 1960; Zeller, Oberländer and Roth, 1968), the distribution of the quantity of label among components may change with plant age; thus the difference in susceptibility of label to catabolism by either soil animals or soil microorganisms. It has been demonstrated before (Kononova, Mishustin and Shtina, 1972) that the mineralization of plant residues in the soil depends on the chemical composition of the plants.

The amount of label respired by the animal and the label eliminated through the faeces are in reasonable agreement with previous studies (Reichle, 1967; White, 1968; Hartenstein, 1964; Hubbell et. al., 1965; Reyes and Tiedje, 1973). The average faecal decomposition by microorganisms inferred from the difference in respiration between treatments with and without soil (23.4% and 32.4% for cottonwood and wheat, respectively) was not very different judging from their independent measurements. Nicholson et. al., (1966) reported a similar degradation percentage (23%) for millipede faecal pellets subject to attack by soil microorganisms.

It now appears that the synergistic role of soil animals and soil microorganisms could be defined at the gut and soil-faecal contact level. The mineralization of plant materials was rapid in the presence of soil microorganisms and soil fauna. Also, the biochemical composition at the substrate and faecal level had an influence on the

susceptibility of materials to degradation.

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Chapter II

EFFECT OF ANTIBIOTICS ON THE GUT MICROFLORA OF AN ISOPOD (Tracheoniscus rathkei Brandt)

Animals without an intestinal microflora would be a useful tool for studying growth, survival and death of the gut microflora and for determining the contribution of the gut flora to the animal's metabolism. Use of antibiotics to reduce the population density of the gut could serve as a convenient alternative to growing and maintaining axenic animals although there are existing methods for the latter (Beck and Stauffer, 1950; Retnakaran and French, 1971). Relative sensitivities of the gut flora to a range of antibiotics could also provide information on the major groups of bacteria present in the gut. Therefore, a variety of antibiotics were examined for their ability to reduce the gut population of woodlice. Using a 10^{-2} dilution from five guts extracted as previously described (Reyes and Tiedje, 1972a), 0.4 ml of the suspension was spread on the surface of predried (2 da) nutrient agar plates. Separate antibiotic sensitivity discs (BBL, Cockeyville, Maryland) containing tetracycline (30 µg), chlortetracycline (30 µg), oxytetracycline (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), lincomycin (2 µg), dihydrostreptomycin (10 µg), penicillin G (10 units),

polymixin B (300 units), and bacitracin (10 units) were pressed lightly on the surface of the agar. Discs were variously positioned on different plates to detect any synergistic combinations. The plates were equilibrated in a refrigerator at $5^{o}C$ for 2.5 hr, then incubated for 24 hr and finally evaluated for growth inhibition.

The data in Table 1 show that the broad spectrum antibiotics, the tetracyclines, produced the greatest inhibition of growth of the platable gut flora. To minimize chances of creating antibiotic toxicity to the animal, only two of the most effective antibiotics, tetracycline and chlorotetracycline, were chosen for use in feeding studies.

To test the effectiveness of this combination, five randomly selected animals were dissected and the gut contents diluted and the microflora plated on nutrient agar as previously described (Reyes and Tiedje, 1974a). A second group of five animals was fed with 10 µl of a mixture of 500 ppm each of tetracycline and chlorotetracycline absorbed in 6 mm^3 propylene oxide sterilized wood chips. The gut contents were plated, following the above procedure, two days after the initial feeding. The untreated animals contained 5.8×10^7 platable organisms per gut while the antibiotic treated animals contained only 3.7×10^4 organisms per gut, or approximately a 1000-fold reduction in population density. This reduction should be sufficient to serve as a control for experiments to determine the contribution of the gut flora in plant residue digestion (Reyes and Tiedje, 1974b). These

Table 1. Effect of antibiotic on growth of the platable population from the woodlouse gut as determined by sensitivity discs.

Antibiotic	Growth of gut flora*
Tetracycline	0
Chlortetracycline	0
Oxytetracycline	0
Chloramphenicol	+1
Erythromycin	+2
Lincomycin	+3
Dihydrostreptomycin	+1
Penicillin G	+3
Polymixin B	+2
Bacitracin	+3

*Growth response scale: 0 = no growth to +3 = no inhibition

studies have shown that the animal's metabolism of previously assimilated carbon is unaffected by the antibiotic treatment.

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Chapter III

ECOLOGY OF THE GUT MICROFLORA OF AN ISOPOD (*Tracheoniscus rathkei* Brandt)

BACKGROUND

The balance of our dynamic terrestrial ecosystem depends on nutrient recycling and rate of energy flow mediated by its biotic constituents. In previous studies, woodlice and soil microorganisms were shown to be more efficient in degrading ^{14}C-labeled yeast (Reyes and Tiedje, 1973) and in degrading ^{14}C-labeled plant materials (Reyes and Tiedje, 1974a) than each separately. Since one possible site of animal-microbial interaction in organic matter decay is the intestinal tract, this study was conducted to assess the significance of the gut microflora in the metabolism of plant residue as well as to evaluate the composition and ecology of the microflora. Hopefully, this will contribute to the integrated biological approach of studying nutrient flow in terrestrial ecosystems.

MATERIALS AND METHODS

Soil animals

Tracheoniscus rathkei Brandt, a terrestrial isopod commonly found in northern forests, was collected from campus woodlots and reared in the laboratory on a diet of decomposing wood also found at the collection site. Other rearing details have been previously described (Reyes and Tiedje, 1973).

Estimation of microbial population

The gut of the animal was aseptically extracted by holding the head of the animal with sterile fine tipped forceps and pulling the last abdominal segment with another forceps; this operation was done in sterile plastic Petri dishes in a laminar air flow hood. The removed gut (not including the hepatopancreas) was macerated in 1 ml of sterile distilled water containing five glass beads (3 mm diameter) vigorously shaken with a vortex mixer for 1 min. Leaf or wood chip samples were homogenized in the same manner but for 3-5 min depending on the size. The aqueous suspension was serially diluted up to 10^{-5} times in sterile distilled water prior to pour plating in nutrient agar and/or in leaf extract agar. There were two plates/dilution/sample. Leaf extract agar was prepared using 1.5% agar, 0.1% glucose and 1% stock extract solution obtained from 20 g (dry weight) of freshly collected leaf litter autoclaved with 500 ml of

distilled water for 15 min at 15 psi. Nutrient agar gave the highest colony counts among the several media tested and thus was used routinely for most experiments.

For samples that needed expression of results in microbial count per unit weight of sample, 0.1 ml of the macerated sample was pipetted into a preweighed 1 cm-diameter aluminum foil disc that had been heated to 110°C to constant weight and cooled in a desiccating jar. The sample was dried at 60°C for 24 hr and weighed on a microelectrobalance (Cahn Instrument Co., Paramount, Cal.). The weight of glass from attrition during maceration was corrected by subtracting the dry weight of 0.1 ml from test tubes that contained water only. In the case of samples expressed per weight of gut contents, an additional correction was made by subtracting the mean weight of macerated gut wall obtained from gut samples in which the contents were previously dislodged by gentle shaking.

Laboratory rearing and the gut flora

Ten animals were sacrificed at each sampling period and the platable microbial population determined in the manner described above. The sampling periods occurred after days 1, 10, 41 and 50 in the laboratory and the plating media were nutrient agar and leaf extract agar.

Starvation and the gut population

Fifteen animals were allowed to feed for 24 hr on wood materials collected from the forest. Subsequently, the animals were transferred to sterile Petri dishes without food and incubated inside a plastic box lined with moist filter paper. Five animals were sacrificed after 12, 36 and 72 hr of incubation and the microbial population of the gut contents determined by the above plate count procedure.

Replica plating

The fraction of gut flora that was able to grow anaerobically when initially grown aerobically or was able to grow aerobically when first grown anaerobically was determined by the replica plating technique. The samples that were initially incubated anaerobically were prepared under strict anaerobic conditions using the Hungate anaerobic technique (Hungate, 1969). Freshly autoclaved distilled water to be used in serial dilutions was cooled, 0.05% cysteine was added as a reductant and the tubes were sealed with rubber stoppers. The test tubes were ascertained to be anaerobic by running a parallel control tube which contained 0.004% rezazurin as a redox potential indicator (-0.61 mv). The samples were then surface plated on a prehardened Brewer anaerobic agar (Difco Laboratories, Detroit, Mich.) which had equilibrated for 48 hr inside an anaerobic plastic glove box (Coy Manufacturing Co., Ann Arbor, Mich.) described by

Arankani et. al. (1969). Anaerobiosis inside the chamber was indicated by the rezazurin indicator present in Brewer anaerobic agar. After 3 days, plates with approximately 30 colonies were replica plated onto another Brewer anaerobic agar plate and a nutrient agar plate inside the anaerobic box; the plates were incubated aerobically at 25°C. A parallel experiment was conducted but samples were first incubated aerobically on nutrient agar plates. Replica plating was done onto Brewer anaerobic agar and nutrient agar plates as before but the plates were incubated anaerobically inside the glove box.

Microbial growth in the gut and faeces

The platable microbial count of leaf discs randomly cut with No. 6 cork borer from leaf litter samples was determined. The gut and fresh faeces were also assayed for microbial counts using five randomly selected animals. Autoclaved (25 min at 15 psi) and non-autoclaved leaf discs were fed to another five animals and the microbial count of the uneaten food, the gut contents and faeces were determined after three or more faeces had been excreted by the animals.

Fate of microorganisms in the gut

Colonies that frequently occurred during plating were isolated for reintroduction experiments to determine the fate of bacteria in the gut of the soil animal. Two isolates which were common in the gut and had easily recognizable

colonies were identified to be species of Pseudomonas and Flavobacterium. They were grown in 100 ml of medium containing yeast extract, 1 mg; glucose, 50 mg; K_2HPO_4, 160 mg; KH_2PO_4, 40 mg; NH_4NO_3, 50 mg; $MgSO_4 \cdot 7H_2O$, 20 mg; $CaCl_2 \cdot 2H_2O$, 2.5 mg; and $FeCl_3 \cdot 6H_2O$, 2.5 mg. In the case of Flavobacterium, the medium contained 250 µC of ^{14}C-uniformly labeled glucose.

The cells were harvested by centrifugation and washed three times with cold sterile distilled water. The cells were resuspended to a final concentration of 158 X 10^4 cells of Flavobacterium per µl (0.002 µC/µl) and 232 X 10^4 cells of Pseudomonas per µl.

Dry propylene oxide sterilized wood chips (6 mm^3) that had absorbed 5 µl each of Pseudomonas and labeled Flavobacterium (0.01 µC) suspensions were individually fed to five animals that had received an antibiotic mixture of 5 µg each of chlortetracycline and tetracycline (Reyes and Tiedje, 1974b). The antibiotics were carried in 10 µl of sterile distilled water absorbed in wood chips. The respired, faecal, and assimilated labels were measured after 1 week by the method used in a previous study (Reyes and Tiedje, 1973). The unconsumed label in the chip was determined. The microbial counts in the gut, faeces and wood chip were also determined.

Wood chips with 10 µl of sterile water added and fed to animals served as the control.

Animal respiration and the gut flora

Four animals that had been fed non-labeled cottonwood leaves (Populus deltoides) which contained the antibiotic mixture described above were starved for 1, 2 and 4 days. After each starvation period, each animal was allowed to feed on 2.5 mg of ^{14}C-labeled cottonwood and the respired label and the gut population were measured 24 hr after feeding.

RESULTS

The gut flora

The platable gut population remained rather constant during a 50-day rearing period in the laboratory when fed natural food (Table 1). The constancy of the population is also indicated by the fact that a similar percentage of the nutrient agar population grew on leaf extract agar throughout the 50-day period.

Table 2 shows that a significant portion - an average of 51% - of the gut population was facultative anaerobes. There was a corresponding increase in the percentage of population that was able to grow anaerobically as the aerobic population decreased from 75.7 to 8.3 X 10^8/g dry wt of gut contents, suggesting that the stable gut flora was capable of anaerobic metabolism. No obligate anaerobes as determined by this procedure were apparent since all colonies recovered from the initial anaerobic incubation also grew aerobically.

Table 1. Effect of laboratory rearing on the microbial composition of the woodlouse gut.

Time in captivity (days)	Microorganisms/gut X 10^{-3}*		% on leaf extract
	Nutrient agar	Leaf extract	
1	112.7±35	20.6±2	18.3
12	260.4±36	65.2±15	25.1
41	128.6±82	31.1±14	24.2
50	221.7±50	48.9±20	22.1
Average	180.9±72	41.5±17	23.0

*Mean of ten animals

Table 2. Physiological type of microorganisms found in the gut of woodlice.

Animal	Plate count/g dry wt of gut contents X 10^{-8}*		
	Aerobic	Anaerobic	% Anaerobic
A	75.7	6.2	8.2
B	58.1	16.8	28.9
C	17.7	14.1	79.8
D	8.3	7.3	87.5
Average	40.0	11.1	51.1

*When the initial condition was anaerobic, the average count was 7.1 X 10^5/g dry wt, but were all able to grow aerobically.

As shown in Table 3, the population decreased 60-fold during a 72 hr starvation period. Since the data is expressed as population/g dry wt of gut contents, it indicates that the population relative to gut residue has dropped sharply probably due to lysis and assimilation.

Microbial changes from food to faeces

Table 4 shows the platable count of microorganisms per gram of material. The increase in population density from leaf to gut to faeces indicates growth. In addition the larger population increase after feeding leaf materials also suggests microbial growth during passage through the animal. When the animals were fed autoclaved leaves, the population between leaf and faeces increased 600-fold. Fungi, which had reinfested autoclaved leaves, were also found in the gut but had disappeared from the faeces. Significantly, the major bacterial colony types were similar in the gut and faeces but were absent from the leaf material.

Fate of microorganisms in the gut

When a dominant gut and faecal bacterium, Flavobacterium, was reintroduced in wood chip material as uniformly-^{14}C-labeled viable cells, the majority of the cells were metabolized as indicated by the high percentage of respired and assimilated label (Table 5). The respired $^{14}CO_2$ could have resulted from Flavobacterium respiration, respiration of other gut flora and animal respiration.

Table 3. Effect of starvation on the microbial population of woodlouse gut.

Time after last feeding (hr)	Count/g dry wt of gut contents* X 10^{-8}
12	41.4±23.1
36	4.2±2.8
72	0.7±0.4

*Mean of five animals

Table 4. Microbial density of leaf, gut and faeces during passage through woodlice.

Treatment	Plate count/g dry wt X 10^{-8}*		
	Leaf	Gut contents	Faeces
As collected	2.8±1.0	6.3±2.4	103.8±43.2
After feeding			
Natural leaves	2.4±1.4	16.2±4.6	401.1±85.3
Autoclaved leaves	1.1±0.5 (2.3±0.9)**	4.8±2.1 (3.0±1.2)	671.9±88.7 (0)

*Mean of five samples

**Values in parenthesis are fungal colony counts from the same plates as the bacterial counts.

Based on ingested label and recovered label in the uneaten food, the expected population in the gut and faeces was 12.6 and 54.5 X 10^4, respectively. These values were very close to the actual count (Table 5). In contrast the population of the wood chip declined 3-fold during the week incubation period. The simplest explanation is that the remaining label is contained in viable cells and that other viable and non-viable cells have been digested. Table 6 provides the recovery values for both isolates and the total population compared to the natural population present in the control. In the antibiotic treated (inoculated) animals it is apparent that both inoculated species and naturally reinfesting species followed the same pattern. The final total population values for the inoculated animals compares favorably with the values for the non-inoculated animal. Particularly for the inoculated animal, the trend of higher counts in the food and lower counts in the faeces suggests digestion of bacteria in the gut.

Respiration vs. gut population

A positive correlation between respiration and gut flora during the first day and a negative one after 2 and 4 days indicates that microflora may initially be aiding the animals during digestion (Figure 1). However, the rapid decline in gut flora accompanied by increase in respiration during starvation indicated greater reliance of the animal on the plant material.

Table 5. Fate of ^{14}C-labeled Flavobacterium fed to woodlice for one week.

Fate	% of ingested label	Plate count X 10^{-4}*	
		Expected	Actual
Respired	40.3	--	--
Assimilated	32.9	--	--
In gut	5.0	13	9±6
In faeces	21.8	55	51±6
Wood chip**	--	385	117±11

*Mean of five animals

**48.7% of the original label remained in the wood chip after the experiment (non-ingested label).

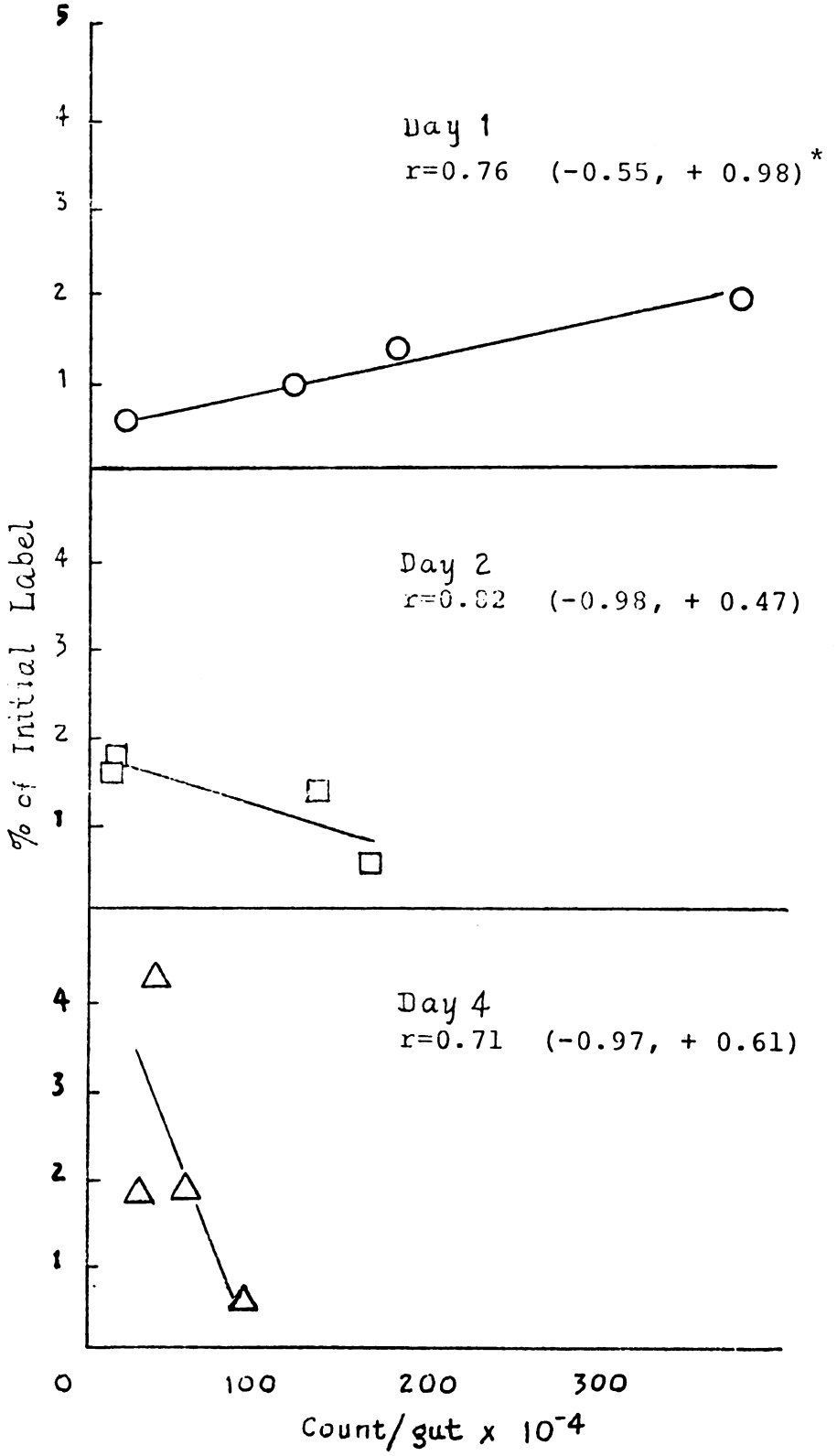
Table 6. Recovery of isolates and natural population fed to woodlice for one week.

Source	Not-inoculated Total population	Inoculated with isolates*		
		Total population	Pseudomonas	Flavobacterium
	Plate count X 10^{-4}**			
Gut	27±11	61±15	34±12	9±6
Faeces	626±134	279±43	157±42	51±6
Wood chip	696±126	643±70	515±34	117±11

*Inoculum contained 1162 X 10^4 Pseudomonas cells and 790 X 10^4 Flavobacterium cells absorbed in wood chips.

**Mean of five animals

Figure 1. Correlation between respiration and gut population after antibiotic treated animals were starved for 1, 2 and 4 days. Note - *indicates 95% confidence interval. When zero is included, evidence is not sufficient to claim significant correlation ($P < 0.05$).


Day 1
r=0.76 (-0.55, + 0.98)*
Day 2
r=0.82 (-0.98, + 0.47)
Day 4
r=0.71 (-0.97, + 0.61)
% of Initial Label
Count/gut x 10^{-4}
0
100
200
300
1
2
3
4
5

DISCUSSION

Litter is known to be colonized by microorganisms from the budding stage of the living plant parts (Leben, 1972) up to the time the plant parts fall to the ground (Hering, 1972; Holm and Jensen, 1972; Seidler et. al., 1972; Swart, 1972). Although the succeeding interactions among microbial populations of these plant materials will determine the resulting populations prior to grazing by soil animals, establishment of the organisms in the tissues before incorporation into the soil (Bruehl and Lai, 1966) and inoculation of the plant residue upon contact with soil and litter (Bandoni and Koske, 1974) both play a role in the colonization of litter materials. The successful microbial species in the colonized litter, however, would not be expected to be the successful species in the gut because of the difference in the two environments. The fates of the microbial species entering the gut would be growth, lysis and elimination. Those that established themselves in the gut could maintain a quasi-steady-state population depending on their rate of multiplication, availability of substrate, rate of wash-out and susceptibility to digestive enzymes. Since a more or less constant and unique population was present in the gut during a 50-day rearing period in the laboratory, such a steady-state population of resident flora is suggested. Considering the 99% reduction in population during starvation, favorable growth conditions are necessary if the gut

flora is to maintain itself against digestion and elimination. Other evidence that microorganisms may be multiplying in the gut was the increase in density of bacteria during the transfer from leaf to gut to faeces, both in freshly collected and laboratory fed animals. The presence of fungi in autoclaved leaves indicates fungal recolonization in the absence of a bacterial population due to contamination from the animal. The absence of fungi in the faeces suggests digestion by the animal. In laboratory experiments, Finstein and Alexander (1962) were able to show that bacteria could outcompete fungi when carbon was not limiting. The animals before feeding had lower numbers of microorganisms in the gut and the increase could not have been due to mere addition of organisms from non-autoclaved leaves because of lower microbial density in that material. In addition, the difference of major colony types in the food compared with the gut and faeces suggests that there was digestion and growth of different flora at the same time.

Digestion of organisms in the gut is indicated by the results of starvation experiment. There might be a mechanism that triggers the enzymes to be more active under conditions where gut contents have low available energy, possibly a lower optimum pH as observed for proteinases in guts of other soil animals. Woodlice are known to rest intermittently for 24-48 hr periods after feeding (Cole, 1946) to allow more time for digestion of food. Singh (1971) observed that there was less bacteria and an absence of protozoa in collembola

after starvation when compared to feeding animals. Faecal cultures showed that some bacteria and fungi were able to pass through the gut; the fungi were ingested primarily in the spore form. Fredeen (1964) found that blackfly larvae developed to adults when fed only with washed suspensions of bacteria.

Two bacteria, Pseudomonas and Flavobacterium, that were isolated and reintroduced into the animals represent common genera found in plants and organic residues at various stages of decomposition (Holm and Jensen, 1972; Last and Warren, 1972; Greaves, 1973). The respiration of ingested label from the Flavobacterium again gives evidence that a gut resident could be utilized by the animal. The Pseudomonas probably had the same fate as the Flavobacterium according to their relatively similar pattern of distribution in the gut, faeces and uneaten food. The difference between the expected number of recoverable Flavobacterium cells and the actual count observed suggests that digested dead cells were the origin of the respired and assimilated label in the animal. Bayne (1973) made a similar study on land snail Helix pomatia (L.) by injecting ^{14}C-labeled bacteria Serratia marcescens and found that the bacteria declined rapidly inside the snail, especially after starvation. He concluded that the bacterial cells were either phagocytosed or degraded and the label incorporated into the snail tissue. Soil animals, particularly woodlice, do not have such an elaborate digestive system but do have certain digestive

enzymes such as carbohydrases (Rajulu, 1970), cellulase (Kühnelt, 1961; Vonk, 1960), chitinase (Rajulu, 1970; Kühnelt, 1961; Vonk, 1960) and proteinases (Bewley and DeVilles, 1968). In addition, ultrastructural studies of hepatopancreas of woodlice revealed the secreting nature of the cells lining the interior wall of the organ (Donadey and Besse, 1972; Clifford and Witkus, 1971).

When the relation of gut population to animal respiration is considered, it also appears that the general decline with time in the respired label from starved antibiotic treated animals was due to consumption. Although the reliability of the data is low, this experiment does suggest that early during starvation, the animal respired less label because there were more bacteria in the gut available for digestion. But later when the population diminished due to digestion resulting from further starvation and lack of microbial substrates, the animal had to depend more on the given food than on the existing gut flora for energy. An average T. rathkei weighing 35 mg would require 46 cal/day for maintenance at 20°C (White, 1968). Based on the average calorific value of the bacterial cells at 5383 cal/g ash free dry weight (Pochazka, 1970), the animal would need 8.5 mg of bacteria per day to maintain itself. Even though a 73% digestion efficiency of Flavobacterium by the woodlice was high, this would still require a total of 11.6 mg of bacterial cells, a value impossibly high for the gut of the animal. Compared to a leaf litter food source (4625 cal/g)

and 33% digestion efficiency, the animal would require 30 mg of food for the same energy. With a bacterial concentration of 5.5 X 10^4/mg of leaf litter (Bärlocher and Kendrick, 1973), 30 mg of litter would contain 16.5 X 10^4 cells. This value compares favorably with our result of 2.4 and 2.8 X 10^8/g leaf. This population would not provide a significant amount of the energy needs of the animal.

The importance of facultative anaerobes in the gut is consistent with expected low oxygen conditions due to restricted gas transport and active respiration. The absence of obligate anaerobes suggests that the gut is not constantly anaerobic which is also consistent with the simple tube-like nature of the gut of woodlice and much different from the more differentiated digestive systems of termites (Breznak et. al., 1973), and crane fly larvae (Klug and Kotarski, 1974) and boll weevils (Bracke and Markovetz, 1974) which have hind guts containing obligate anaerobes. In a study of lepidopteran larvae, Kingsley (1972) found that the gut inhabitants were facultative heterotrophs and that the number of aerobes and anaerobes fluctuated relative to each other. Similarly, the microbial population in the midgut of Simulium damnosum larvae, which ranged from 8.9 X 10^4 - 400 X 10^4, were found to be aerobic (Burton, Perkins and Sodhi, 1973).

The presence of freshly macerated organic material in the moist intestinal tract of a soil animal would seem to be a favorable environment for microbial growth. This hypothesis is supported by the present study. However, it is also

apparent that the animal is capable of digesting the microflora and assimilating the contents. Thus, a dynamic turnover of microbial biomass occurs. The relative contribution of the microbial activities to organic matter digestion by the animal appears to be minimal, however, the utilization of microbial biomass may be important particularly during periods of animal starvation. In the terrestrial world of soil animals where adverse conditions often threaten the animal's survival, the contribution of the microbial gut flora could be vital.

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