THE DISTRIBUTION OF ANAEROBIC BACTERIA ALONG A SOIL DRAINAGE CATENA

by

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Introduction

The geometry of the soil matrix provides a variety of potential microhabitats where soil anaerobes can survive or proliferate. Anaerobic bacteria have been isolated from a variety of soils in different climatic areas (Smith and Gardner, 1944; Hungate, 1950; Rodriguez-Kabana, Jordan and Hollis, 1965; Ottow, 1971; Rice and Paul, 1972; Thayer, 1974; Smith, 1975). Soil moisture can have a direct effect on the metabolism of these bacteria, or it can affect them indirectly by changing aeration (Baver, Gardner and Gardner, 1972; Patrick and Delaune, 1972; Howeler and Bouldin, 1971), composition of inorganic nutrients (Patrick and Delaune, 1972), organic matter (Alexander, 1961) and pH (Patrick and Reddy, 1975). It was the objective of this study to determine the quantity of strictly anaerobic or facultative bacteria in soil along a drainage catena in which moisture was a major variable. This is one of the first investigations where the soil was exposed to minimal amounts of oxygen from the time of sampling until incorporation into an anaerobic culture tube. Furthermore, this is also one of the first investigations comparing a variety of media to determine which medium would yield maximum recovery of anaerobes. An enumeration of the strictly anaerobic bacteria in soil cannot prove that the metabolism of these bacteria has a definite influence upon soil nutrient recycling, yet the presence of large numbers of vegetative cells of strict anaerobes indicates that the activities of this population must be considered in any metabolic balance sheet of a soil ecosystem.

Materials and Methods

Research Site and Soil Parameters. The study area featured a soil drainage catena near Blacksburg, Virginia, which extended from a perpetually poorly drained meadow soil with a predominant cover of annual and perennial plants to a well drained forest soil with a predominant mixed hardwood cover. The catena extended approximately 7 meters from the wettest to the dryest sites. Three study sites were examined: a poorly drained site, an intermediate drained site, and a well drained site. Undisturbed soil cores were taken from these 1 square meter plots with a 4 inch diameter corer. To minimize oxygen contamination, these large cores were wrapped in plastic bags and transported to the laboratory. The plots were sampled during the period of December 27, 1975 to September 11, 1976. Chemical analyses were performed on air dried subsamples of this soil. Organic matter was determined by heating the soil to 350-400 C for 8 hr. Direct bacterial counts were determined using a modification of the acridine orange method of Strugger (1949) and a Lietz #301 microscope.

Preparation of Anaerobic Dilutions. Soil samples were analyzed for viable anaerobes within 3 hr after removal of the core from the ground. One gram subsamples were selected from the 7-10 cm soil depth in the center of the core and the soil was placed immediately into tubes containing 9 ml of anaerobic dilution fluid (Holdeman and Moore, 1973). The sampling zone was arbitrarily chosen because it corresponded to the apparent perpetually anaerobic zone in the wettest site, an extensively gleyed area in the intermediate site and a well aerated region in the

well drained site. All inoculations and dilutions were conducted under a flowing stream of oxygen free CO_2 and followed protocol suggested by the VPI & SU Anaerobe Lab (Holdeman and Moore, 1973). All anaerobic media were prereduced and contained 0.05% cysteine hydrochloride (Difco, Detroit, Mich.) and 0.0001% resazurin (Difco) as a reducing agent and E_h indicator, respectively.

Enumeration of Anaerobe Population. The quantity of soil anaerobes was determined by employing a 5 tube Most Probable Number (MPN) technique using prereduced liquid media prepared according to VPI & SU Anaerobe Lab procedures (Holdeman and Moore, 1973). Growth was assayed by visual turbidity, and presumptive negative tubes at high dilutions were examined microscopically as a confirming check on absence of growth. The following media were used: brain heart infusion (BHI), trypticase soy, cooked meat, cooked meat plus 0.5% glucose, peptone-yeast extract-mineral salts (PY), PY-soil extract (PYS), and PY-soil extract-sand (1 gm coarse sand per culture tube) (PYSS). All of the above media were prepared in proportions found in the VPI & SU Anaerobe Lab Manual (Holdeman and Moore, 1973) with the following exceptions: 1) the trypticase soy was prepared according to Difco Laboratories (Detroit, Mich.); 2) the peptone-yeast extract based media were prepared with 0.1% peptone and 0.1% yeast extract; and 3) the soil extract was prepared according to Babiuk and Paul (1970). All tubes were incubated at 28 C for two or three weeks. All counts were expressed on the basis of soil dry weight.

Enumeration of Anaerobic Sporeforming Population. The same procedure used in the MPN determinations was followed, with the exception that the 10^3 dilution blanks, after inoculation, were heat shocked at 80 C

for 10 min prior to subculture into broth media.

Enumeration of Obligate Anaerobe Population. A roll tube counting technique and BHI roll tubes (Hungate, 1950) were utilized. A 0.1 ml aliquot of soil dilution fluid was added to the BHI roll tube, which was tempered at 43-50 C, and the tube was spun for approximately 10 min until the agar hardened. Three replicate tubes were prepared for each soil dilution and they were incubated at 28 C for three weeks before the colonies were counted. A dissecting microscope was used to observe the colonies. Up to 13 colonies were picked from each soil subsample. It was often necessary to streak these colonies several times on BHI roll tubes following the VPI & SU Anaerobe Lab procedure in order to obtain a pure culture. Once a culture was judged by colonial and microscopic characteristics to be pure, it was inoculated into a BHI-$MnCl_2$ (10^{-5}M) anaerobic broth. After 48 hr of incubation at 28 C, stab cultures from these tubes were made into BHI agar deep slants and incubated for 48 hr at 28 C under aerobic conditions. The growth pattern in the butt and slant of these deeps was used to judge the strict or facultative nature of the culture. All cultures which were strict anaerobes were checked for catalase production by mixing a drop of 3% H_2O_2 with a loop of culture under a dissecting microscope. The number of strict anaerobes in the anaerobic population of the soil was determined by dividing the number of strict anaerobes by the number of anaerobes examined and then multiplying this number by the roll tube count.

Obligate Anaerobe Spore Forming Determination. All cultures which were determined to be obligate anaerobes were subcultured from BHI-$MnCl_2$ after four days growth into the peptone-yeast extract-starch broth of

Holdeman and Moore (1973). After inoculation the starch broths were heat shocked at 80 C for 10 min before incubating at 28 C for 48 hr under anaerobic conditions. Growth in this medium indicated the culture was an obligately anaerobic sporeformer.

Results

Some of the soil characteristics of the 8 cm depth of the study site are shown in Table 1. Over the nine month period of the study, which included the relatively mild winter of 1975-76, there were marked differences in soil moisture quantity between the different soil drainage sites. The poorly drained and intermediate soils were similar in pH and organic matter and were favorable for bacterial growth in terms of soil reaction and available substrate, while the acidic nature of the well drained soil was typical for a forest soil, and less organic matter had accumulated on this soil. No attempt was made to examine the quantity of fungi on all sites, but the low pH in the forest soil may have created conditions more favorable for fungal decomposition than bacterial activity. The differences in the microscopic bacterial count between the three sites were small; the direct microscopic count was between 1 to 4 x 10^{10} bacteria/g dry weight soil on the three sites. Since microscopic counts may include vegetative cells, dead cells, and various spore bodies, a substantial discrepancy between microscopic and viable counting methods was expected.

The anaerobic MPN counts observed in cultures of soil from the poorly drained site during the nine month study period are shown in Table 2. The highest recovery of anaerobes was obtained with the complex medium, cooked meat plus 0.5% glucose. This medium yielded a count which was one to two fold greater than the other media but four orders of magnitude less than the microscopic count. Therefore, the differences are too small to make absolute comparisons about the superiority of any

Table 1. Study Site Soil Characteristics (8 cm depth).

Plot Drainage Character	Moisture[a] %	pH	Organic Matter %	Direct Microscopic Count (x 10^{10}/gdws)
Poorly Drained	112.37[b]	7.1 (4-16-76) 6.6 (9-11-76)	15.78	2.1
Intermediate	39.18[c]	6.9 (4-16-76) 6.2 (9-11-76)	15.07	3.2
Well Drained	18.69[c]	5.0 (4-16-76) 4.2 (9-11-76)	3.03	1.9

[a]Gardner, 1965.

[b]Mean of 22 samples between 12-27-75 and 9-11-76.

[c]Mean of 18 samples between 1-29-76 and 9-11-76.

Table 2. Anaerobe Count (x 10^6/gdws) from the 8 cm Soil Depth of the Poorly Drained Site Using Different Media with the MPN Anaerobic Culture Technique after 14 Days Incubation at 28 C.

Sample Date	Trypticase Soy	BHI	Cooked Meat	Cooked Meat + 0.5% Glucose	PY	PYS	PYSS
12-27-75	0.9	2.5	--	--	--	--	--
1-15-76	--	3.1	0.5	--	--	--	--
6-3-76[a]	--	2.5	--	8.8	0.5	3.5	2.9
8-2-76[a]	--	3.9	--	4.5	1.3	2.3	1.8
8-28-76[a]	--	1.9	--	4.2	0.3	1.2	0.9

[a]Mean of two subsamples obtained from one core.

one medium, but the data does support the hypothesis that the complex rich organic media were more successful than the low substrate-soil extract media in recovering soil anaerobes. The fact that soil extract yielded higher counts than the same medium without soil extract indicates that this component may be important in the nutrition of some soil anaerobes.

The anaerobic MPN counts for the three soils on three different sampling dates are shown in Fig. 1. These data support the hypothesis that more anaerobes were present on the wetter soils of the drainage catena. The perpetually wet site had 1-3 logs more anaerobes than the well drained site.

The seasonal differences observed on the poorly drained soil are shown in Fig. 2. The spring enumeration yielded the highest number of anaerobes whereas the winter and summer values were very similar.

The counts of obligate anaerobes of the three soils are shown in Table 3. A mean count of 1.4×10^6/g dry weight soil was observed on 11 September 1976. Since approximately 2×10^6/g dry weight soil were observed with the MPN method only a few weeks earlier (August 28, 1976--Fig. 2), it may be concluded that a large portion of the anaerobes observed was strictly anaerobic. Furthermore, approximately 50 percent of the isolates picked from the BHI roll tubes were strict anaerobes. The intermediate soil had a count of obligate anaerobes, which was approximately one log less than the poorly drained plot, and the well drained plot had an obligate anaerobic count of several logs less than the poorly drained. The intermediate soil is at the interface of oxidizing and reducing zones and the variable data observed for that soil

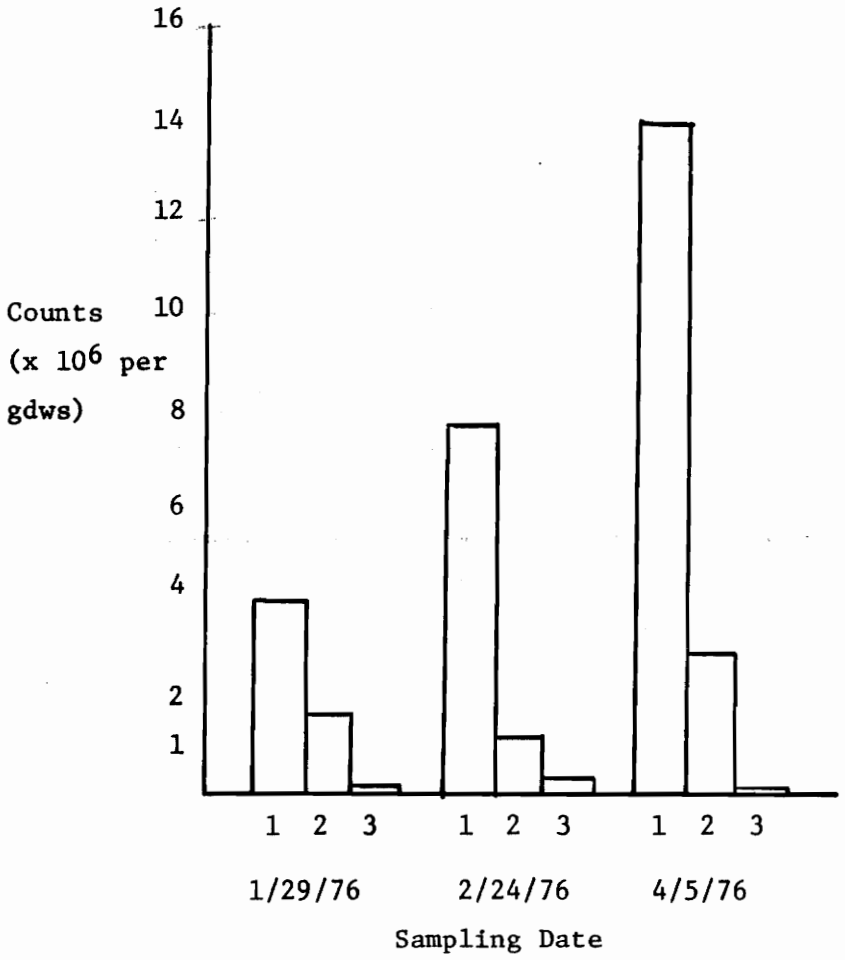

Figure 1. Counts of anaerobes (facultative and obligate) in soil of different drainage character using BHI medium and the MPN technique. 1--Poorly Drained. 2--Intermediate. 3--Well Drained sites.

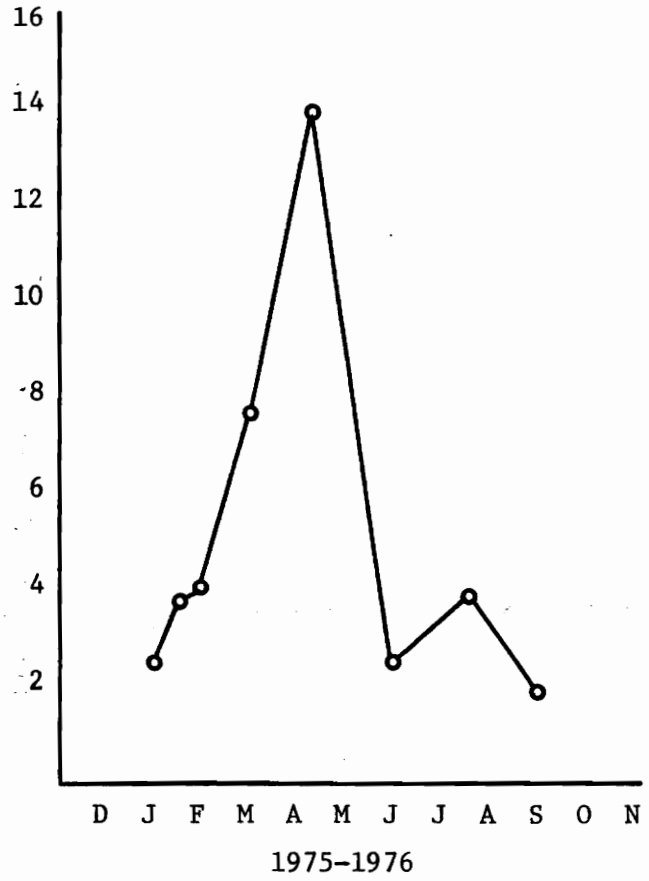

Figure 2. Seasonal count of anaerobes (facultative + obligate) in the poorly drained soil using BHI medium and the MPN technique. All values represent a single determination excepting those obtained on the May-August sampling which are the mean of two determinations.

Table 3. Counts of Obligate Anaerobes from Soil of Different Drainage Character Using BHI Anaerobic Roll-Tube Technique.[a]

Plot Drainage Character	Count of Obligate Anaerobes (x 10^6/gdws)[b]	Mean For Each Plot
Poorly Drained	core 1 - 1.5	1.4
	core 2 - 2.0	
	core 3 - 0.8	
Intermediate	core 1 - <0.6	<0.7
	core 2 - 1.3	
	core 3 - 0.3	
Well Drained	core 1 - <0.01	<0.07
	core 2 - <0.05	
	core 3 - 0.15	

[a]11 September 1976.

[b]Each value represents the mean of determinations on two sub-samples from each core.

in Table 3 was expected. A comparison of the anaerobe MPN count shown in Fig. 1 and the obligate anaerobe count shown in Table 3 indicates that a substantial portion of the anaerobes in the intermediate soil may be facultative in regard to their oxygen metabolism.

The number of endospore forming anaerobes observed in the three soils are shown in Table 4. The intermediate plot had a higher anaerobic endospore forming bacterial population than the wetter or dryer sites on two April sample dates. This apparent discrepancy was due to the fact that the endospore forming population on the intermediate site was composed primarily of facultative Bacillus spp. On the 11 September sampling, out of 63 isolates subcultured from the intermediate site, no strictly anaerobic spore formers were detected. On this same date, 0.59×10^6/g dry weight soil of strictly anaerobic endospore forming bacteria were observed on the poorly drained soil. The population of strictly anaerobic endospore formers on the well drained site can be conservatively estimated to be 1 to 2 logs less than the population of strictly anaerobic endospore formers on the poorly drained site.

Table 4. Enumeration of Facultative and Strictly Anaerobic Endospore containing bacteria (x 10^6/gdws) from the poorly drained soil after two weeks of incubation on brain heart infusion medium at 28 C.

Plot Drainage Character	No. Anaerobic Endospore Forming Bacteria[a]		No. Strictly Anaerobic Endospore Forming Bacteria[b]
	4-14-76	4-19-76	9-11-76
Poorly Drained	1.1	4.6	0.45
Intermediate	2.1	18.0	N.D.[c]
Well Drained	0.095	0.4	<0.04

[a]Heat shocked MPN technique.

[b]Determined from portion of flora observed on BHI roll tube isolation method which can survive heat shock after transfer from BHI-MnCl broth to PY starchbroth.

[c]Not detected.

Discussion

The well known discrepancy between direct microscopic counts and viable plate counts of microorganisms in soils has been reviewed and discussed (Russell, 1973). The enumeration of soil microbial populations has little application to the goal of understanding the metabolism of these organisms *in situ* without information on growth, death, and metabolic rates of the population. The techniques to study *in situ* soil metabolism are still in the developmental stages, and two of the principle tools of the contemporary microbial physiologist and ecologist, the direct microscopic count and cultural count, yield radically high values in the former case and conservatively low values in the latter when soil is examined. We hypothesized that one of the reasons for the low recovery of viable forms from soil was the failure of soil microbiologists to approach the isolation of soil anaerobes with the rigorous oxygen free systems used by investigators in the study of rumen and intestinal contents. A modification of the Hungate-VPI & SU Anaerobe Lab technique was used in this study to demonstrate that a very abundant population of strict anaerobes did exist in a poorly drained soil, but the population values obtained (10^6/g dry weight soil) were too low to support the idea that strict anaerobes are a large portion of the observed microscopic population (10^{10}/g dry weight soil). Some of the media tested in this study were more successful than others, yet the differences of anaerobe recovery with different media were small. The use of gas mixtures other than 100% CO_2 and the incorporation into the media of fermentation end-products (short chain fatty acids), cytochromes, catalase,

etc. may increase the recovery of anaerobes. The use of soil extract appears to be a significant factor in the growth of some soil anaerobes. The use of microaerophilic conditions rather than oxygen free conditions may permit the isolation of organisms which have previously been isolated in high numbers from soil (Casida, 1965). Our data support the observation of Smith (1975) that a complex medium such as BHI will yield a higher recovery of soil anaerobes than a dilute medium such as peptonized milk. Smith observed that cooked meat medium with 0.5% glucose was an excellent medium to isolate soil clostridia, and that medium was the most successful of the media tested in this study.

The range of strictly anaerobic gram positive sporeforming rods obtained from different soil sites (0.07-1.4 x 10^6/g dry weight soil) were similar to other values reported in the literature. Gibbs and Freame (1965) employed their 'Differential Reinforced Clostridial Media' which is highly selective for the clostridia, and obtained from various soil samples of unspecified origin, counts in the range of 0.035-1.2 x 10^6/g dry weight soil. Thayer (1974) employed BBL anaerobic agar with incubation under prepurified nitrogen and obtained counts in the range of 10^5/g wet soil from relatively dry Texas prairie soils, while Smith (1975) used a cooked meat plus 0.5% glucose medium and obtained counts in the range of 0.00027-3.3 x 10^6/g dry weight soil from various soil specimens. It is difficult to determine from these studies which medium or isolation technique is the most efficient in the isolation of strict anaerobes, due to the fact that a variety of soils were examined using various methods of sampling. The medium and incubation conditions we employed were similar to those of Smith (1975), but his soil samples

were air dried and therefore selective for the clostridia. Smith found all of his obligate anaerobes to be clostridia, while we found only one third of our strictly anaerobic isolates from the poorly drained site to be confirmed members of this group. This indicates that careful handling of the soil samples during the transition from field to laboratory may have permitted the isolation of bacteria not previously reported by other workers.

The population of total anaerobes,[1] obligate anaerobes, and obligate endospore forming anaerobes along the drainage catena were negatively correlated with the degree of soil aeration. The decrease of total soil anaerobes from the poorly drained to well drained soils (employing the MPN technique) was not as great as the decrease of obligate anaerobes (employing the roll tube technique). The facultatively anaerobic bacteria clearly dominated the anaerobic population on the intermediate and well drained soils. Even on the poorly drained site the facultative anaerobes constituted a substantial portion of the anaerobic flora. The intermediate soil was probably depleted of oxygen to the same extent as the poorly drained soil for much of the year, but during the drier periods the facultative bacteria may have established themselves in microhabitats where oxygen, however transitory, was available. The populations of anaerobes observed on this soil catena were consistent with the idea that microenvironments were available in soil for the growth of anaerobes, because 1) obligate anaerobes were isolated from the soil of

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[1]This term refers to all bacteria capable of metabolizing anaerobically and includes facultative and obligate anaerobes.

even the well drained site and 2) there was possibly a substantial population of nonspore forming anaerobes in the poorly drained soil. Higher total populations of anaerobes might have been observed if the soil had been sampled at a point nearer the soil surface where microbial activity is more dynamic, but the 8 cm depth was selected because it was hypothesized that a greater portion of microflora would be obligate anaerobes at that depth. Similar soil depths have been used by other workers. In light of these data we must conclude that the very high numbers of bacteria observed by microscopic counts are composed of: microaerophiles, resistant resting forms, bacteria that grow very slowly on the type of culture media provided in this study, dead forms, or a combination of these factors.

Previous investigations have stressed the importance of Clostridium spp. as the dominant strict anaerobic genus in soil (Smith, 1975). Of the strictly anaerobic isolates cultured in this study, less than one third were gram positive sporeforming rods. One possible reason is that a large number of the isolates were clostridia which lacked the ability to sporulate under the culture conditions used or were highly pleomorphic in cell morphology and gram reaction. Another possibility is that a sizeable population of strict anaerobes not belonging to the genus Clostridium exists in soil. It will require extensive taxonomic data on these isolates to resolve this problem. Many of the sporeforming anaerobes observed on the intermediate drainage soil were apparently facultative Bacillus spp. The diverse nature of the anaerobic population on all three soils makes it impossible to estimate the physiologically active portion without further experimentation, such as has been done

in aquatic habitats employing radioactive isotopes (Molongoski and Klug, 1976).

The population of total anaerobes observed on the poorly drained soil during the nine month period of the study was relatively constant with the important exception of the late winter and early spring values. The sharp rise of the population during that period may have coincided with substrate increases associated with death of plant and microbial cells during the winter and the return of warmer soil temperatures. A rhizosphere effect could have contributed to this increase, but this does not appear likely, considering the depth of soil examined throughout the study.

Howeler and Bouldin (1971) have noted that the diffusion rate of oxygen in soil micropores will be slow, and consequently these micropores should represent microhabitats which are anaerobic especially if the microorganisms present are physiologically active. It is also likely that the concentration of inorganic electron acceptors such as nitrate, sulfate, etc. would be quickly exhausted and the accumulation of end-products would quickly reduce the metabolic rate of the microflora even when available substrate has not been exhausted. Microenvironments in soil (Alexander, 1964) may explain the presence of obligate anaerobes on well drained sites such as the well-drained site in this study. However, the facultative bacteria appeared to be more successful competitors than the obligate anaerobes for the microhabitats in the intermediate and well drained soils examined.

Conclusions

1) The population of strict anaerobes observed on a soil drainage catena varied from 10^6, 10^5, 10^4/g dry weight soil on the poorly drained, intermediate, and well aerated soils respectively.

2) The population of total anaerobes observed on a soil drainage catena varied from 10^6, 10^6, 10^5/g dry weight soil on the poorly drained, intermediate, and well aerated soils respectively.

3) The population of total anaerobes on the poorly drained soil was relatively constant over a nine month period with the exception of an increase in early spring.

4) The gram positive sporeforming rods constituted roughly one third of the total obligately anaerobic soil microflora on the poorly drained soil. Some strict anaerobes were isolated on the well aerated soil; these forms either represent resting forms of cells metabolizing in a primarily anaerobic microhabitat in the soil. The isolation of significant numbers of apparently non-spore forming bacteria indicates this group should receive further examination.

5) The use of the Hungate-VPI & SU anaerobic isolation technique with undisturbed soil samples yielded counts of obligate anaerobes similar to other values reported in the literature although the numbers of non-clostridial strict anaerobes isolated by this technique were much higher than other values reported in the literature. This may reflect the fact that soil contains a population of non-clostridial strict anaerobes which has not, as yet, been examined, due to failure of other less rigorous techniques to culture this component of the anaerobic

population.

6) The cooked meat plus 0.5% glucose medium gave the highest recovery of soil anaerobes of the various media tested. However, the increase was less than one log over the other media. Similar anaerobic recoveries were observed with complex media such as brain heart infusion and dilute media such as peptone-yeast extract-soil extract.

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APPENDICES

Appendix 1. Counts of anaerobes[a] [illegible] soil on different drainage character using [illegible] technique.

Plot Drainage Character	Sampling Date		
	1/29/76	2/24/76	4/5/76
Poorly Drained	4.1[b]	7.7	14.0
Intermediate	1.7	1.2	2.9
Well Drained	0.17	0.28	0.096

[a]Counts x 10^6/g dry weight soil.

[b]Each value represents one determination.

Appendix 2. Seasonal count of anaerobes (facultative + obligate) in the poorly drained soil using BHI medium and the MPN technique.

Sample Date	Count (x 10^6/g dry weight soil)
12/27/75	2.5
1/15/76	3.8
1/29/76	4.1
2/24/76	7.7
4/5/76	14.0
5/27/76	2.5[a]
7/18/76	3.9[a]
8/28/76	1.9[a]

[a]Each value represents the mean of two determinations.

Appendix 3. Obligately anaerobic culture gram-stains.

Type	Number	%
Poorly Drained Site--28 Isolates		
Gram + sporeforming rods	9	32.1
Gram + rods	11	39.3
Gram + coccobacilli	2	7.2
Gram + sporeforming coccobacilli	2	7.2
Gram variable rods	1	3.6
Gram - rods	3	10.7
Intermediate Site--10 Isolates		
Gram + sporeforming rods	Not detected	--
Gram + rods	5	50
Gram - rods	5	50
Well Drained Site--6 Isolates		
Gram + sporeforming rods	1	16.7
Gram + rods	2	33.3
Gram + coccobacilli	1	17.0
Gram + sporeforming coccobacilli	2	33.3

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(ABSTRACT)

Strict anaerobic culture techniques were used to enumerate the anaerobic bacteria present in three soil sites located along a drainage catena near Blacksburg, Virginia. An anaerobic cooked meat plus 0.5% glucose medium cultured the largest number of anaerobes from the poorly drained soil. The population of obligate anaerobes ranged from 10^6/ g dry weight soil on the poorly drained soil (% moisture = 112.06) to 10^5/g dry weight soil on the intermediate soil (% moisture = 34.51) to 10^4/g dry weight soil on the well drained soil (% moisture = 20.81). The population of organisms able to grow anaerobically (facultative plus obligate) ranged from 10^6/g dry weight soil on the poorly drained site to 10^5/g dry weight soil on the well drained site. This same population on the poorly drained site was relatively constant over a nine month period with the exception of a sharp rise in early spring. The clostridia constituted at least one third of the obligately anaerobic bacteria present on the poorly drained soil. A sizeable percentage of the obligate anaerobic isolates on this site were either clostridia which formed spores unable to germinate in the medium employed, clostridia which were very pleomorphic in cell shape and gram reaction, or nonsporeforming obligate anaerobes. These results indicate that strict anaerobes and possibly nonsporeforming strict anaerobes exist in soils

of different drainage character even though facultative organisms appear to be more successful competitors on the more well drained sites.