DOE/ER/60991--T3

DISCLAIMER

Physiologically Anaerobic Microorganisms of the Deep Subsurface

Final Performance Report

June 1, 1990 - August 31, 1993

S. E. Stevens, Jr. and K.-T. Chung

Department of Biology

Memphis State University

Memphis, Tennessee 38152

October 1993

Prepared for the U.S. Department of Energy
under Grant Number DE-FG05-90ER60991

Herein, I summarize the progress made by the research team assembled at Memphis State University on our DOE sponsored project titled "Physiologically Anaerobic Microorganisms of the Deep Subsurface" (DOE Contract No. DE-FG05-90ER60991).

SCHEDULE OF MAJOR RESEARCH ACTIVITIES

Year 1 (1990-91)

- Determine numbers, diversity, and morphology of anaerobic microorganisms in 15 samples of subsurface material from the Idaho National Engineering Laboratory (INEL), in 18 samples from the Hanford Reservation (HR) and in 1 rock sample from the Nevada Test Site (NTS); set up long term experiments on the chemical activities of anaerobic microorganisms based on these same samples; work to improve methods for the micro-scale determination of in situ anaerobic microbial activity; begin to isolate anaerobes from these samples into axenic culture; and, begin to identify the axenic isolates.

Year 2 (1991-92)

- Determine selected physiological characteristics of all isolates from INEL, HR, and NTS; after characterization of obligate anaerobes is well started, begin the characterization of facultative anaerobes; compare isolates from different depths at each site; publish fundamental results from the above work.

Year 3 (1992-93)

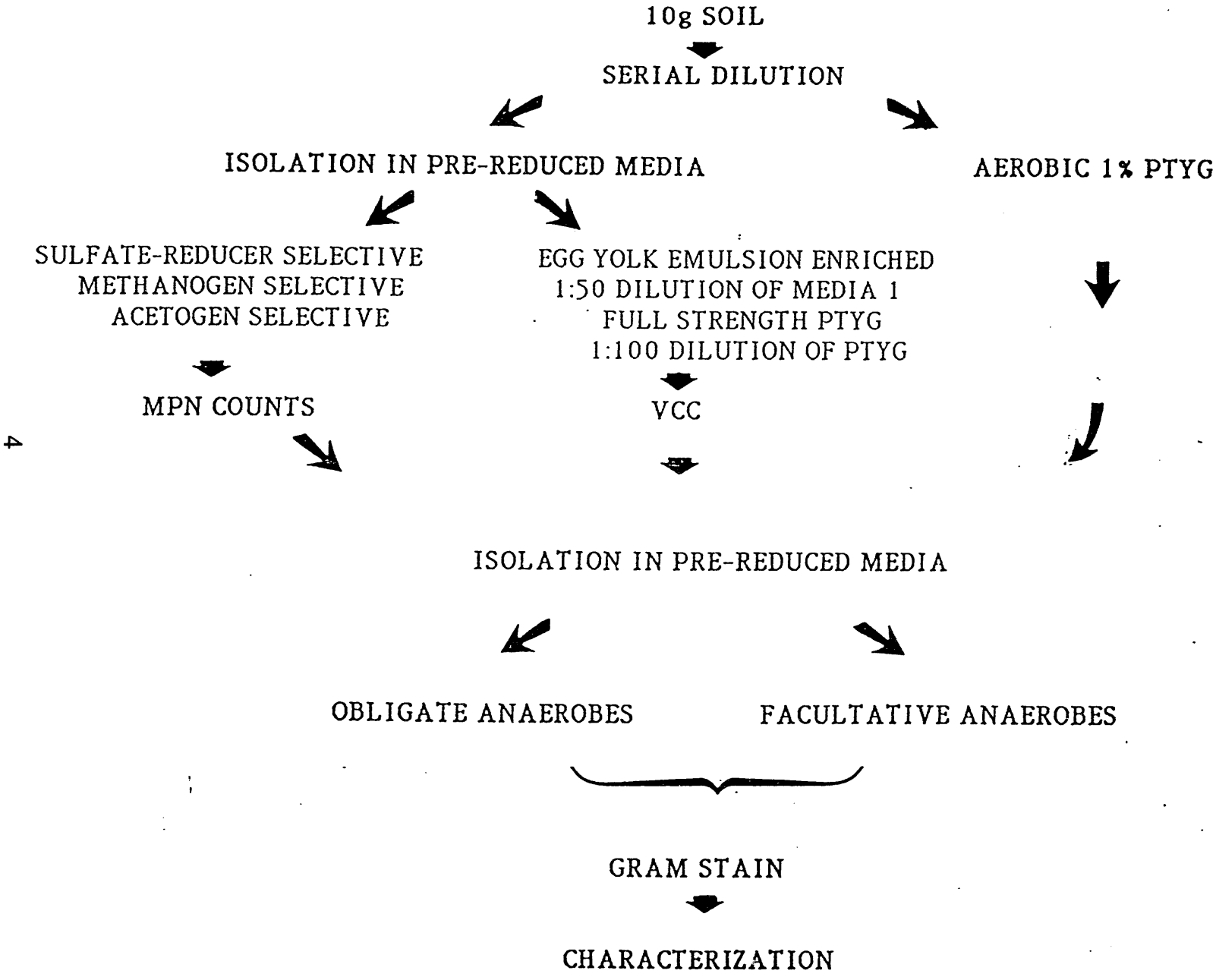
- Look for possible new anaerobic isolates from long term enrichments in stored samples; characterize these as above, if found; begin studies leading to the long term preservation of each axenic culture; analyze data sets from each investigator and determine factors which influence community structure especially in regard to anaerobic microorganisms; transfer preserved isolates to the culture collection at Florida State University; publish additional results.

PROJECT OUTPUT: MAJOR ACCOMPLISHMENTS (6/90 - 7/93)

- We have completed plate counts, MPN's, spin-tube counts, acridine orange direct counts, 4'-6-diamidino-2-phenylindole dihydrochloride, and ATP estimates on 15 samples from the INEL site, 18 samples from the HR site, and 1 sample from the NTS site. A flow chart summarizing sample manipulation is shown in Fig. 1.

  Medium 1 is an egg yolk emulsion medium recommended as a general medium for anaerobic bacteria. Medium 2 is a 1:50 dilution of medium 1. Medium 3 is full strength PTYG. Medium 4 is a 1:100 dilution of medium 3. The primary media, reducing agents, and redox indicators used in the project are described on pages 5-8 of this report. Both spread plates and spin-tubes with pre-reduced (bubbled with 4% H_2 balance $C0_2$) medium were used for counting purposes. The use of PTYG as a growth medium

FIGURE 1


10g SOIL
SERIAL DILUTION
ISOLATION IN PRE-REDUCED MEDIA
AEROBIC 1% PTYG
SULFATE-REDUCER SELECTIVE
METHANOGEN SELECTIVE
ACETOGEN SELECTIVE
EGG YOLK EMULSION ENRICHED
1:50 DILUTION OF MEDIA 1
FULL STRENGTH PTYG
1:100 DILUTION OF PTYG
MPN COUNTS
VCC
ISOLATION IN PRE-REDUCED MEDIA
OBLIGATE ANAEROBES
FACULTATIVE ANAEROBES
GRAM STAIN
CHARACTERIZATION

MEDIA FOR ANAEROBES

1. General Heterotrophs.

MEDIUM I.

COMPOUND	FINAL CONC. g/L medium	STOCK CONC. g/L	ml STOCK/ L medium
[1]Na_2EDTA	0.002	3.0	0.66
[1]A-5 METAL MIX	NA	NA	1.0
[1]Ferric ammonium citrate	0.012	6.0	2.0
K_2HPO_4	0.065	65.0	1.0
KH_2PO_4	0.035	35.0	1.0
Na_2CO_3	0.025	25.0	1.0
NaCl	0.075	75.0	1.0
[1]$MgSO_4$-$7H_2O$	0.075	50.0	1.5
trypticase	15.0	NA	NA
glucose	5.0	NA	NA
yeast extract	1.0	NA	NA
chicken egg yolks	2	NA	NA
[2]BBL granulated agar	15	NA	NA

[1]Stock solutions of these compounds are used in Medium B for cyanobacteria and need not be made up separately.

[2]The agar is added if appropriate.

NA = not applicable.

Milli-Q water should be used in preparing this medium. Measure out about 900 ml of water, add the above listed ingredients, pH the solution to 6.9 as indicated below, and then make to 1L with the required amount of water. If agar is to be added, it is added after the solution is made to 1L. Likewise with reducing agents and redox indicators.

The pH of the medium should be adjusted to 6.9 prior to autoclaving.

Reducing agents and Redox indicators are prepared separately and added to the medium just prior to use.

Medium II.

Medium II is a 1:50 dilution of Medium I in Milli-Q water.

Medium III.

Medium III is full-strength PTYG medium.

COMPOUND	FINAL CONC. g/L medium
Peptone	5.0
Tryptone	5.0
Yeast extract	10.0
Glucose	10.0
$MgSO_4$-$7H_2O$	0.6
$CaCl_2$-$2H_2O$	0.07
BBL granulated agar	15.0

MEDIUM IV.

Medium IV is a 1:100 dilution of the first four ingredients of Medium III.

COMPOUND	FINAL CONC. g/L medium
Peptone	0.05
Tryptone	0.05
Yeast extract	0.1
Glucose	0.1
$MgSO_4$-$7H_2O$	0.6
$CaCl_2$-$2H_2O$	0.07
BBL granulated agar	15.0

2. Specific anaerobes

MEDIUM V, for ennumeration and isolation of sulfate reducing bacteria.

COMPOUND	FINAL CONC. g/L medium	STOCK CONC. g/L	ml STOCK/ L medium
*KH_2PO_4	°-5	50.0	10.0
NH_4Cl	1.0	none	weigh
[1]$CaSO_4$-$2H_2O$	1.26	none	weigh
[2]$MgSO_4$ -$7H_2O$	2.0	none	weigh
#sodium lactate	3.5	60% syrup	4.7
Yeast extract	1.0	none	weigh
Ascorbic acid	0.1	none	weigh
Thioglycollic acid	0.1	none	weigh
FeS04 7H20	°-5	none	weigh
BBL granulated agar	15.0	none	weigh

Use building distilled water. Adjust to pH 7.5 with NaOH after boiling to dissolve agar.

Use ml indicated in column 3 from medium A stocks in Rm 521.

#Use Sigma DL-Lactic Acid, catalog number L-1375.

[1]Revised medium substitutes $CaCl_2$-$2H_2O$, 0.67 g

[2]Revised medium substitutes $MgCl_2$-$7H_2O$, 2.0 g

REDUCING AGENTS AND REDOX INDICATORS FOR ANAEROBIC MEDIA

The following reducing agents will be used as appropriate in Media I - IV for the viable cell counts of general heterotrophic anaerobic bacteria.

Reducing agent	E_o' [mV]	Preparation of stock solution	Final conc. in medium
Sodium thioglycollate	<-100	1% (w/v) in MQ[1] H_2O, autoclaved and stored under O_2 free argon.	0.05%
Na_2S-$9H_2O$	-225	1.2% (w/v) Na_2S-$9H_2O$ in MQ H_2O, autoclaved and stored under O_2 free argon.	0.025%
Cysteine	-340	1% (w/v) in MQ H_2O, autoclaved and stored under O_2 free argon.	0.025%
Titanium (III) citrate	-480	5 ml of 15% (w/v) solution of $TiCl_3$ added to 50 ml of 2.36% (w/v) sodium citrate dihydrate and neutralized by addition of saturated Na_2CO_3. Sterilized with 0.22μm filters. NOTE: must be used within 1 day at a concentration of 30ml/L medium!	0.5-2mM

REDOX INDICATOR.

Sterilize a 0.1% aqueous stock solution of resazurin by passing it through a 0.22μm filter, and add to the growth medium to give a final concentration of 0.0003%. NOTE: resazurin is not needed when titanium (III) citrate is used as reducing agent!

Resazurin (blue) $\xleftarrow{\text{pH <6.5}}$ Resorufin (oxidized) (pink) $\xleftarrow{E_o' \text{ -51 mV}}$ Resorufin (reduced) (colorless)

Diluent or medium is useful only if colorless!

for anaerobic bacteria was validated by plating pure cultures of anaerobic bacteria obtained from the ATCC on both their ATCC recommended medium and on PTYG. The results support the use of PTYG as a plating medium for anaerobes (see Table 1). In addition, we used Postgate's medium to determine the presence of sulfate reducing bacteria by MPN, and found none. We essentially made two changes in this medium (the revised recipe is given on page 7) and used it to successfully culture sulfate reducing bacteria from several core subsamples from INEL and HR.

We also used 2 media, H_2-CO_2 and methylamine-acetate respectively to determine the presence of methanogens and acetogens in INEL, HR, and NTS samples. The H_2-CO_2 medium contained in 900 ml of deionized water: NH_4Cl, 1 g; $K_2HPO_4 \cdot 3H_2O$, 0.4 g; $MgCl_2 \cdot H_2O$, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.4 g; Na_2-EDTA$\cdot 2H_2O$, 1 ml (500 mg/ml); 1 ml of resazurin (0.1%); 1 ml of trace mineral solution. The trace mineral solution contained per 100 ml of distilled water: $CoCl_2 \cdot 6H_2O$, 150 mg; $MnCl_2 \cdot 4H_2O$, 100 mg; $FeSO_4 \cdot 7H_2O$, 100 mg; $ZnCl_2$, 100mg; $AlCl_3 \cdot 6H_2O$, 40 mg; $Na_2WO_4 \cdot 2H_2O$, 30 mg; $CuCl_2 \cdot 2H_2O$, 20 mg; $NiSO_4 \cdot 6H_2O$, 20 mg; H_2SeO_3, 10 mg; H_3BO_3, 10 mg; $NaMoO_4 \cdot 2H_2O$, 10 mg. The pH of the trace mineral solution was adjusted to 2.5-3.0 with 1N HCl and stored at 4^o C.

TABLE 1.

	CULTURE COUNT (PER ML)		
MEDIUM	*Bacteroides fragilis* ATCC 2374	*Clostridium perfringens* ATCC 3624	*Lactobacillus bulgaricus* ATCC 27558
Reinforced clostridial (oxoid)	9.5×10^8	1.1×10^7	----
	6.8×10^8	1.1×10^7	----
	1.3×10^9	2.9×10^7	----
TPYG	1.2×10^9	1.1×10^7	5.5×10^4
	1.1×10^9	8.8×10^6	6.0×10^4
	1.1×10^9	7.5×10^6	6.0×10^4
Nutrient agar	$< 10^5$	$<10^5$	----
Lactobacillus agar	1.1×10^9	8.0×10^5	1.5×10^5
	7.8×10^8	4.5×10^5	3.2×10^6
	7.7×10^8	3.5×10^5	4.7×10^6
Medium I	No growth	----	4.4×10^5
		----	9.9×10^5
		----	7.9×10^5
Medium II	----	----	2.7×10^5
	----	----	6.5×10^5

All bacteria were grown on BHI for 48 hours.

The mineral salts and trace metal solution was heated to boiling and 1.0 g of yeast extract (Difco) and 1.0 g trypticase peptone (BBL) were added and the solution brought back to a boil. The hot medium was then gassed with O_2 -free CO_2 until it was cool. The CO_2 gas passed through a tangle of hot copper wire that removed the oxygen. After cooling, mercaptoethanesulfonic acid (0.5 g) and $NaHCO_3$ (8.6 g) were added (under the gentle flow of O_2-free gas). The medium was then dispensed (8.9 ml) anaerobically under the gentle flow of 80% H_2/ 20% CO_2 gas mixture into VPI anaerobe tubes that were sealed with #1 butyl rubber stoppers and autoclaved.

The methylamine-acetate medium was prepared using the same mineral salts and trace metal solutions described above. Upon cooling, mercaptoethanesulfonic acid (0.5 g), Na-acetate (4.1 g) and methylamine (1.92 g) were added under the gentle flow of O_2-free nitrogen gas. The medium was dispensed as previously dscribed under the flow of the O_2-free nitrogen gas.

Both autoclaved media were stored at room temperature. Before use, 0.1 ml of 2.5% sterile cysteine-HCl solution was injected into each tube by syringe through the rubber stopper while flushing with sterile O2-free nitrogen gas. The 2.5% cysteine-HCl solution was prepared under a gentle flow of oxygen-free nitrogen gas. The inoculum for these media were 1 ml of the anaerobic PBS 10^{-2} sample dilutions. Samples were incubated at room temperature

for two months. Growth and gas uptake in the H_2-CO_2 medium were indicative of presence of methanogens, and no growth or gas uptake coupled with growth in the methylamine-acetate medium was indicative of the presence of acetogens.

Plate count data, MPN data, and spin-tube counts for 15 samples from INEL and 18 samples from HR are given in Tables 2 and 3. There were no significant differences in plate counts for the INEL samples over the four types of media ($p=0.318$). HR samples did show significantly different ($p=0.003$) results over the four media types. The higher number of positive plates and the greater growth on these plates compared to the INEL plates probably accounts for this difference. Both sites tended to have the lowest plate counts on medium 1 and the highest counts on medium 4. MPN results were similar at both sites with the greatest depths having the highest aerobic counts.

ATP estimates for these same samples are not given because none of them, with the exception of the spiked sample from INEL, indicated a reliably significant concentration of ATP. The NTS sample provided by Dr. Penny Amy did not show any anaerobic growth. Methane production occurred in two HR samples and gas was taken up in an additional HR sample. These results indicate methanogenic bacteria in samples 0007b and 0307 and a probable acetogenic bacterium in sample 0407 from HR. AODC data are plotted in Figs. 2 and 3 as number of cells versus depth. Total cell

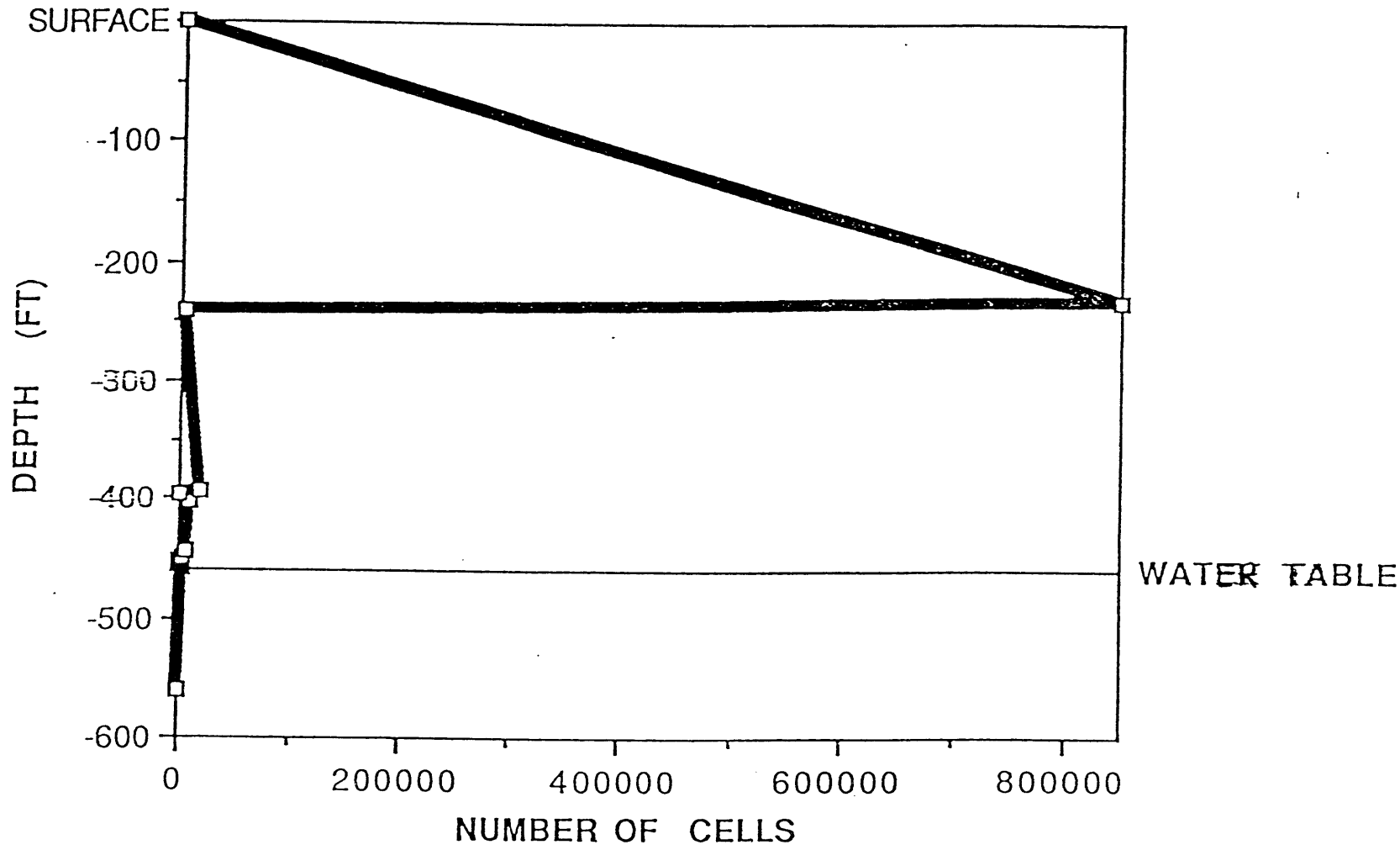
TABLE 2. INEL

Sample #	I PC- O_2	II PC- O_2	III PC- O_2	IV PC- O_2	SO_4 - RED	PR- O_2	III - O_2 TUBES	SO_4-RED TUBES
0-4-6	<0.5	<0.5	<0.5	<0.5	<0.5	>7.0	3.2	<0.5
207	<0.5	NG	<0.5	<0.5	NG	>7.0	NG	<0.5
240/5	<0.5	<0.5	<0.5	<0.5	NG	<2.0	NG	NG
394/1-7	<0.5	<0.5	<0.5	<0.5	NG	<2.0	NG	NG
395/6-8	<0.5	<0.5	<0.5	<0.5	NG	<2.0	NG	NG
400/9-11	<0.5	NG	<0.5	NG	NG	<2.0	NG	NG
443/8-11	NG	<0.5	<0.5	<0.5	NG	<2.0	<0.5	NG
448/0-3	NG	NG	NG	NG	NG	<2.0	-	-
451/10-452/10	NG	NG	<0.5	NG	NG	<2.0	NG	NG
452/1-453/1	4.8	<0.5	<0.5	<0.5	NG	-	<0.5	NG
452/11-453/1	<0.5	NG	<0.5	NG	NG	>7.0	NG	<0.5
455/10-456/5	<0.5	<0.5	NG	<0.5	NG	>7.0	<0.5	NG
458/6-459	<0.5	<0.5	<0.5	<0.5	NG	>7.0	<0.5	NG
520/2	NG	NG	NG	NG	NG	>7.0	-	-
560-564	<0.5	<0.5	<0.5	<0.5	NG	>7.0	NG	NG

TABLE 3. Yakima Barricade Borehole

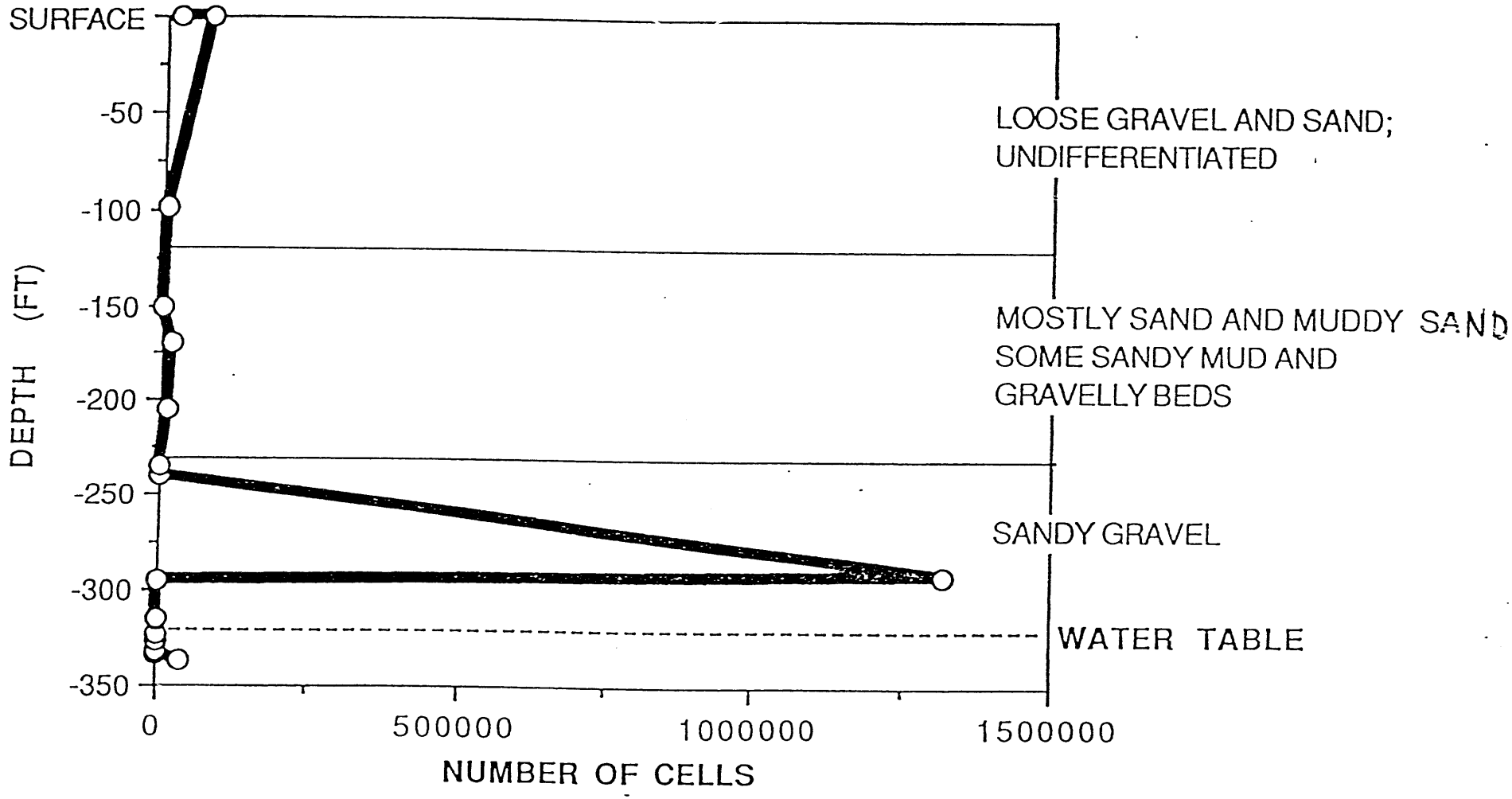
Sample #	I PC- O_2	II PC- O_2	III PC- O_2	IV PC- O_2	SO_4 - RED	PR - O_2	III - O_2 TUBES	SO_4-RED TUBES
HMB-1	<0.5	<0.5	<0.5	3.6	<0.5	>4.0	<1.0	<1.0
0007A	NG	4.1	<0.5	4.2	<0.5	>7.0	4.2	<1.0
0007B	NG	<0.5	NG	4.5	<0.5	8.1	2.6	<1.0
107	<0.5	<0.5	<0.5	<0.5	NG	>7.0	NG	NG
207	NG	<0.5	NG	5.9	<0.5	>7.0	NG	NG
307	<0.5	<0.5	<0.5	<0.5	NG	4.4	NG	NG
407	NG	NG	NG	NG	NG	4.3	NG	NG
507	NG	NG	NG	NG	NG	<2.0	NG	NG
537	NG	NG	NG	NG	NG	4.7	NG	NG
607	4.1	4.9	5.0	4.3	NG	3.8	NG	NG
707	NG	<0.5	<0.5	2.5	NG	<2.0	NG	NG
807	NG	NG	NG	NG	NG	<2.0	NG	NG
907	NG	NG	NG	NG	NG	<2.0	NG	NG
1007	NG	NG	<0.5	<0.5	NG	<2.0	NG	NG
1107	4.7	NG	NG	NG	NG	<2.0	NG	NG
1207	5.5	5.6	5.3	5.6	NG	<2.0	NG	NG
1307	NG	4.0	<0.5	<1.0	NG	>4.0	NG	NG
1407	NG	5.1	<0.5	4.8	NG	<2.0	<1.0	NG

FIGURE 2



CELLS PER ML VS. DEPTH FOR INEL SAMPLES AS DETERMINED BY ACRIDINE ORANGE STAINING.

FIGURE 3



CELLS PER ML VS. DEPTH FOR HANFORD SAMPLES AS DETERMINED BY ACRIDINE ORANGE STAINING.

counts in all but a few samples were very low, ranging from 0 to 10 total cells over 25 fields. However, the highest numbers of cells were between 200 and 300 feet below the land surface at both INEL and HR.

- We have recorded the colonial morphology, cellular morphology, and Gram reaction on each colony type observed on plates and/or spin-tubes. These results are tabulated in Tables 4 and 5. The Gram reaction and morphological results also give a preliminary notion of diversity. Pie charts summarizing cell type results are presented in Figs. 4 and 5. About 85% of the isolates from INEL were Gram + while only about 67% of those from HR were Gram +. The main difference between these sites is in the number of rod-type cells, 22% at INEL and 6% at HR. Several of these Gram + isolates were isolated from long term enrichments and were very slow in appearing. Though coccobacilli were the predominant morphological type at both INEL and HR, 6% of total were Gram - at INEL and 20% at HR. Gram - bacteria and those with a rod-type cellular morphology were minority representatives at both INEL and HR. Also for both sites the following associations were noted: Gram + cell types and spindle-shaped, small, white, opaque colonies; Gram - cell types and circular-shaped, large, white, transparant colonies.

TABLE 4. INEL

Sample #	Gram Stain	Cell Type	Cell Number	Colony Shape	Colony Color	Colony Elevation	Colony Size
0-4-6							
0-4-6A S	+	cb	pairs?	spindle	wo	dbl convex	small
0-4-6A L	+	cb	pairs?	spindle	wo	dbl convex	small
0-4-6B S	+	cb	pairs?	circular	wt	convex	large
0-4-6B L	+	cb	pairs?	circular	wt	convex	large
0-4-6C	-	rod	single	filament	wt	flat	exlarge
207 INEL L	-	rod	single	circular	wt	convex	large
240/5							
240/5A	-	rod	single	circular	wt	convex	large
240/5B S	+	cb	pairs	spindle	wo	dbl convex	small
240/5B L	+	cb	pairs	spindle	wo	dbl convex	small
240/5C S	+	cb	single	?	?	?	?
240/5C L	+	cb	single	?	?	?	?
394/1-7							
394/1-7A S	+	cb	single	spindle	wo	dbl convex	small
394/1-7A L	+	cb	single	spindle	wo	dbl convex	small
394/1-7B S	+	rod	single	circular	wt	convex	large
394/1-7B L	+	rod	single	circular	wt	convex	large
395/6-8							
395/6-8A	+	rod	single	spreading	wt	raised	large
395/6-8B S	+	cb	single	spindle	wo	dbl convex	small
395/6-8B L	+	cb	single	spindle	wo	dbl convex	small
400/9-11							
400/9-11A S	+	rod sh	pairs	circular	wt	convex	large
400/9-11A L	+	rod sh	pairs	circular	wt	convex	large
400/9-11B	+	cb	chains	spindle	wo	dbl convex	small
443/8-11							
443/8-11A	-	rod	single	circular	wo	convex	large
443/8-11B	-	rod	single	spindle	wo	dbl convex	small
448/0-3	No data - sample autoclaved						
451/10-452/10							
451/10-452/10A S	+	cb	pairs	circular	wt	convex	large
451/10-452/10B L	+	cb	pairs	circular	wt	convex	large
451/10-452/10B S	+	cb	pairs	spindle	wo	dbl convex	small
451/10-452/10B L	+	cb	pairs	spindle	wo	dbl convex	small

S-Solidified medium L-Broth medium

TABLE 4. INEL

Sample #	Gram Stain	Cell Type	Cell Number	Colony Shape	Colony Color	Colony Elevation	Colony Size
452/1-453/1							
452/1-453/1A	+	cb	pairs	circular	wo	convex	large
452/1-453/1B	+	cb	single	spindle	wo	dbl convex	small
452/11-453/1							
452/11-453/1A S	+	cb	pairs	circular	wo	convex	large
452/11-453/1A L	+	cb	pairs	circular	wo	convex	large
452/11-453/1B	+	cb	single	spindle	wp	dbl convex	small
455/10-456/5							
455/10-456/5A	-	rod	single	circular	wt	convex	large
455/10-456/5B S	+	cb	pairs	spindle	wo	dbl convex	small
455/10-456/5B L	+	cb	pairs	spindle	wo	dbl convex	small
458/6-459							
458/6-459A S	+	cb	chain	circular	wt	convex	large
458/6-459A L	+	cb	chain	circular	wt	convex	large
458/6-459B	+	cb	chain	spindle	wo	dbl convex	small
520/2	Spiked, did not culture sample						
560-564							
560-564A S	-	rod	chain	circular	wt	convex	large
560-564A L	-	rod	chain	circular	wt	convex	large
560-564B S	+	rod	single	spindle	wo	dbl convex	small
560-564B L	+	rod	single	spindle	wo	dbl convex	small

S- Solidified medium L-Broth medium

TABLE 5. Yakima Barricade Borehole

Sample #	Gram Stain	Cell Type	Cell Number	Colony Shape	Colony Color	Colony Elevation	Colony Size
HMB-1							
HMB-1A S	+	cb	pairs	spindle	wo		small
HMB-1A L	+	cb	pairs	spindle	wo	dbl convex	small
HMB-1B	-	rod	single	circular	wt	convex	large
0007A							
0007A-A S	-	rod	single	circular	wt	convex	large
0007A-A L	-	rod	single	circular	wt	convex	large
0007A-B S	+	cb	pairs	spindle	wo	dbl convex	small
0007A-B L	+	cb	pairs	spindle	wo	dbl convex	small
0007B							
0007B-A S	-	cb	pairs	circular	wt	convex	large
0007B-A L	-	cb	pairs	circular	wt	convex	large
0007B-B S	+	cb	pairs	spindle	wo	dbl convex	small
0007B-B L	+	cb	pairs	spindle	wo	dbl convex	small
0007B-C S	-	cb	pairs	?	?	?	?
0007B-C L	-	cb	pairs	?	?	?	?
0007B-D S	-	cb	pairs	?	?	?	?
0007B-D L	-	cb	pairs	?	?	?	?
107							
107A S	+	cb	pairs	circular	wt	convex	large
107A L	+	cb	pairs	circular	wt	convex	large
107B S	+	cb	pairs	spindle	wo	dbl convex	small
107B L	+	cb	pairs	spindle	wo	dbl convex	small
107C S	+	cb	pairs	irr und	wo	flat	tiny
107C L	+	cb	pairs	irr und	?	flat	tiny
107D S	+	cb	pairs	?	?	?	?
107D L	+	cb	pairs	?	?	?	?
207 Yakima L		rod	both	?	?	?	?
307							
307A	-	rod Lg	single	circular	wt	convex	large
307B S	+	rod Sh	single	circular	wo	flat	tiny
307B L	+	rod Sh	single	circular	wo	flat	tiny
307C	+	rod Lg	single	spindle	wo	dbl convex	small
407							
407A S	-	cb?	pairs?	circular	wt	convex	large
407A L	-	cb?	pairs?	circular	wt	convex	large
407B S	+	cb	pairs	circular	wo	flat	small
407B L	+	cb	pairs	circular	wo	flat	small

S-Solidified medium L-Broth medium

TABLE 5. Yakima Barricade Borehole

Sample #	Gram Stain	Cell Type	Cell Number	Colony Shape	Colony Color	Colony Elevation	Colony Size
507							
507A S	+	cb	pairs	circular	wt	convex	large
507B-(I) S	+	cb	pairs	?	?	?	?
507B-(II) S	+	cb	pairs	?	?	?	?
507B L	+	cb	pairs	?	?	?	?
507A L	+	cb	pairs	circular	wt	convex	large
507A B L	+	cb	pairs	?	?	?	?
537							
537A S	+	cb	pairs	irr und	wo	dbl convex	medium
537A L	+	cb	pairs	irr und	wo	dbl convex	medium
537B S	+	cb?	pairs?	circular	wt	convex	large
537B L	+	cb?	pairs?	circular	wt	convex	large
607							
607A S	-	cb?	pairs?	circular	wt	convex	large
607A L	-	cb?	pairs?	circular	wt	convex	large
607B S	+	cb	pairs	spindle	wo	dbl convex	small
607B L	+	cb	pairs	spindle	wo	dbl convex	small
607C	-	cb	pairs	filamen	wt	flat	exlarge
707							
707A S	+	cb	pairs	circular	wt	convex	large
707A L	+	cb	pairs	circular	wt	convex	large
707B S	+	cb	pairs	spindle	wo	dbl convex	small
707B L	+	cb	pairs	spindle	wo	dbl convex	small
807	No bacteria cultured from sample						
907	No bacteria cultured from sample						
1007							
1007 S	-	cb	pairs	?	?	?	?
1007 L	-	cb	pairs	?	?	?	?
1007(II) L	?	?	?	?	?	?	?
1007A S	-	cb	pairs	circular	wt	convex	large
1007A L	-	cb	pairs	circular	wt	convex	large
1007B S	+	rod Sh	single	irr und	wo	flat	small
1007B L	+	rod Sh	single	irr und	wo	flat	small
1107	No bacteria cultured from sample						
1207	No bacteria cultured from sample						
1307							
1307A	+	cb	pairs	circular	wt	convex	large
1307B	-	rod	single	spindle	wp	dbl convex	
1407							
1407A	+	rod	single	circular	wo	convex	small

S-Solidified medium L-Broth medium

FIGURE 4. INEL - Morphological Types

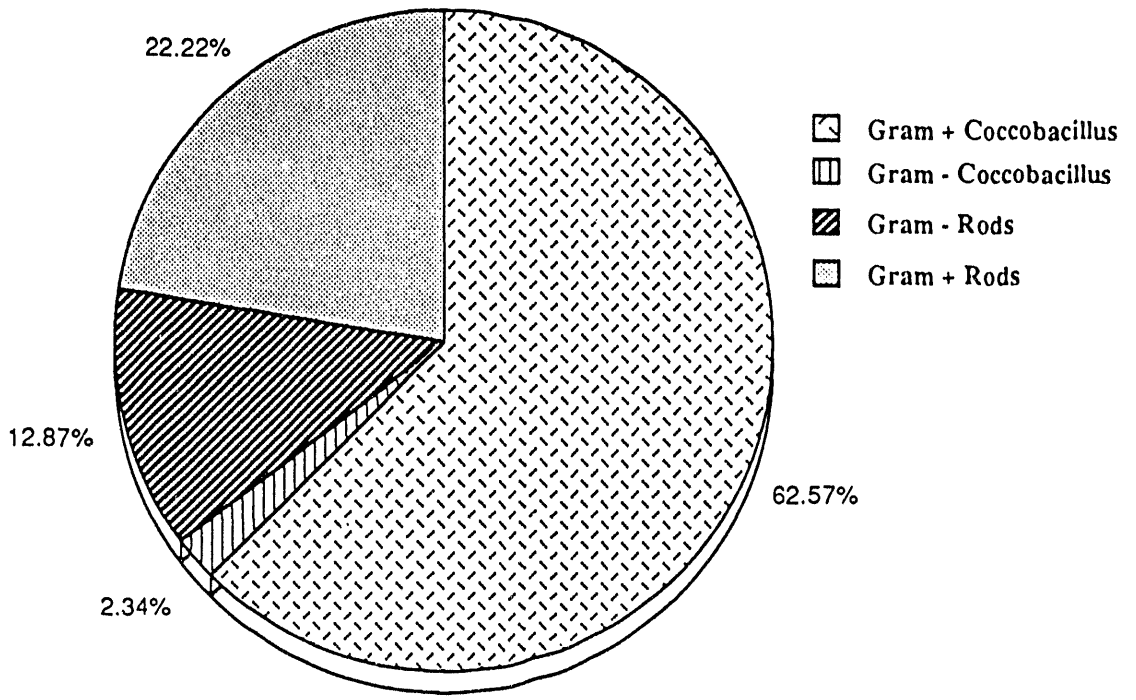
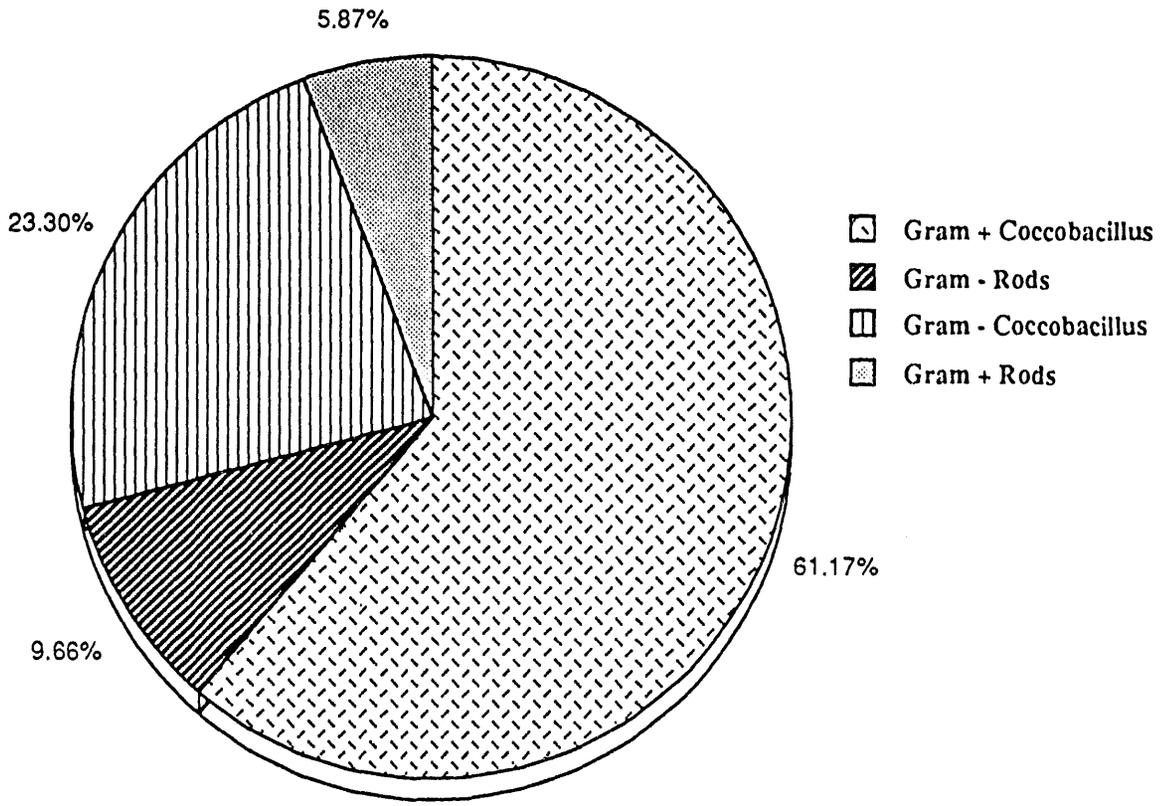


FIGURE 5. Yakima - Morphological Types



- So far, we have isolated into pure culture apparently distinct anaerobes from all the samples examined. Of the 65 pure cultures obtained, 44 are facultative anaerobes and 21 are obligate anaerobes. In addition, we have evidence for sulfate reducing bacteria (positives for 6 different samples), methanogens, and an acetogen although we do not yet have axenic cultures of these.

- We have begun to obtain physiological/biochemical profiles of our axenic cultures. The results of our API physiologocal tests are tabulated in Tables 6 and 7. The physiological tests performed to date afford no unequivocal identifications. However, a number of differences between the two study sites are seen in the percentage of positive results (Table 8). Though most strains could utilize all the carbon sources, glycerol and melizitose responses were positive for less than 50% of the HR strains. Catalase activity and tryptophane activity were significantly different between the two sites. Further characterizations are planned, including the use of *in situ* 16S rRNA hybridization probes on some of the isolates.

- As part of our investigation of factors influencing commuinty structure we have constructed graphs (Figures 6 & 7) of our data on aerobic chemoheterotrophs from HR and INEL according to the specific depth at which the sample was prepared. They are presented as Log_{10} CFU versus depth (m) below the subsurface. Anaerobic relationships to soil sample geologies are presented in

TABLE 6. INEL

Sample #	IND	URE	GEL	ESC	CAT	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE
0-4-6																					
0-4-6A S	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0-4-6A L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0-4-6B S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0-4-6B L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0-4-6C																					
207																					
207A (I) S	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
207A (II) S	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
207A L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
207AB L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
207 INEL S	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+
207 INEL L	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+
240/5																					
240/5A	No API results																				
240/5B S	-	-	-	+	-	+	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+
240/5B L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
240/5C S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
240/5C L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
394/1-7																					
394/1-7A S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
394/1-7A L	No API results																				
394/1-7B S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
394/1-7B L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
395/6-8																					
395/6-8A	No API results																				
395/6-8B S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
395/6-8B L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
400/9-11																					
400/9-11A S		-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
400/9-11A L		-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
400/9-11L	No API results																				

S-Solidified medium L-Broth medium

TABLE 6. INEL

Sample #	IND	URE	GEL	ESC	CAT	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE
443/8-11																					
443/8-11A		No API results																			
443/8-11B		No API results																			
448/0-3		No API results																			
451/10-452/10																					
451/10-452/10A S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
451/10-452/10A L	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
451/10-452/10B S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
451/10-452/10B L	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
452/1-453/1																					
452/1-453/1A		No API results																			
452/1-453/1B		No API results																			
452/11-453/1																					
452/11-453/1A S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
452/11-453/1A L	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+
452/11-453/1B		No API results																			
455/10-456/5																					
455/10-456/5A		No API results																			
455/10-456/5B S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
455/10-456/5B L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
458/6-459																					
458/6-459A S	-	-	-	-	-	+	+	+	+	-	+	-	-	-	+	-	+	-	+	-	+
458/6-459A L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
458/6-459B		No API results																			
520/2		No API results																			
560-564																					
560-564A S	-	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	-	+	+
560-564A L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
560-564B S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
560-564B L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

S-Solidified medium L-Broth medium

TABLE 7. Yakima Barricade Borehole

Sample #	IND	URE	GEL	ESC	CAT	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE
507																					
507A S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
507B (I) S	-	+	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
507B (II) S	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
507B L	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
507A L	No API results																				
507AB L	No API results																				
537																					
537A S	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
537A L	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
537B S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
537B L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
607A S	+	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+
607A L	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
607B S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
607B L		-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
607C	No API results																				
707																					
707A S	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
707A L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
707B S	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
707B L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
807	No API results																				
907	No API results																				
1007																					
1007 S	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
1007 L	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
1007 (II) L	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
1007A S	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
1007A L	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+
1007B S	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
1007B L	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
1107	No API results																				
1207	No API results																				
1307	No API results																				
1407	No API results																				

S-Solidified medium L-Broth medium

TABLE 7. Yakima Barricade Borehole

Sample #	IND	URE	GEL	ESC	CAT	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE
HMB-1																					
HMB-1A S	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
HMB-1A L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HMB-1B	No API results																				
0007A																					
0007A-A S	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
0007A-A L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0007A-B S	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0007A-B L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
007B																					
0007B-A S	-	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+
0007B-A L	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
0007B-B S	+	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+
0007B-B L	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
0007B-C S	+	-	-	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+
0007B-C L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0007B-D S	-	-	-	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+
0007B-D L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
107																					
107A S	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
107A L	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
107B S	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
107B L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
107C S	-	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+
107C L	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
107D S	+	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+
107D L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
207 Yakima L	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+
307																					
307A	No API results																				
307B S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
307B L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
307C	No API results																				
407																					
407A S	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
407A L	-	-	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
407B S	-	-	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+
407B L	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

S-Solidified medium L-Broth medium

TABLE 8. PERCENTAGE OF ANAEROBIC ISOLATES SHOWING VARIOUS METABOLIC ACTIVITIES

METABOLIC TRAITS	INEL (n=18)	HR (n=27)
Esculin hydrolysis	83.33	74.07
Only esculin positive[1]	55.56	25.93
Catalase activity	27.78	74.07
Tryptophane metabolism	11.12	40.74
Catalase & Tryptophane[2]	5.56	40.74
Gelatin liquefaction	5.56	7.41
Urease activity	0	3.70
ANAEROBIC ASSIMILATION OF:		
Glucose	100	100
Mannitol	100	100
Lactose	100	100
Saccharose (Sucrose)	94.44	100
Maltose	94.44	70.37
Salicin	94.44	70.37
d + Xylose	94.44	88.89
l + Arabinose	88.89	100
Glycerol	83.33	37.04
Cellobiose	100	96.30
Mannose	94.44	100
Melezitose	83.33	51.85
Raffinose	72.22	96.30
Sorbitol	94.44	100
Rhamnose	83.33	100
Trehalose	100	100

[1] Esculin was positive, but there was no catalase or tryptophan activity

[2] Both catalase and tryptophane activity were positive

FIGURE 6. INEL CFUs BY DEPTH

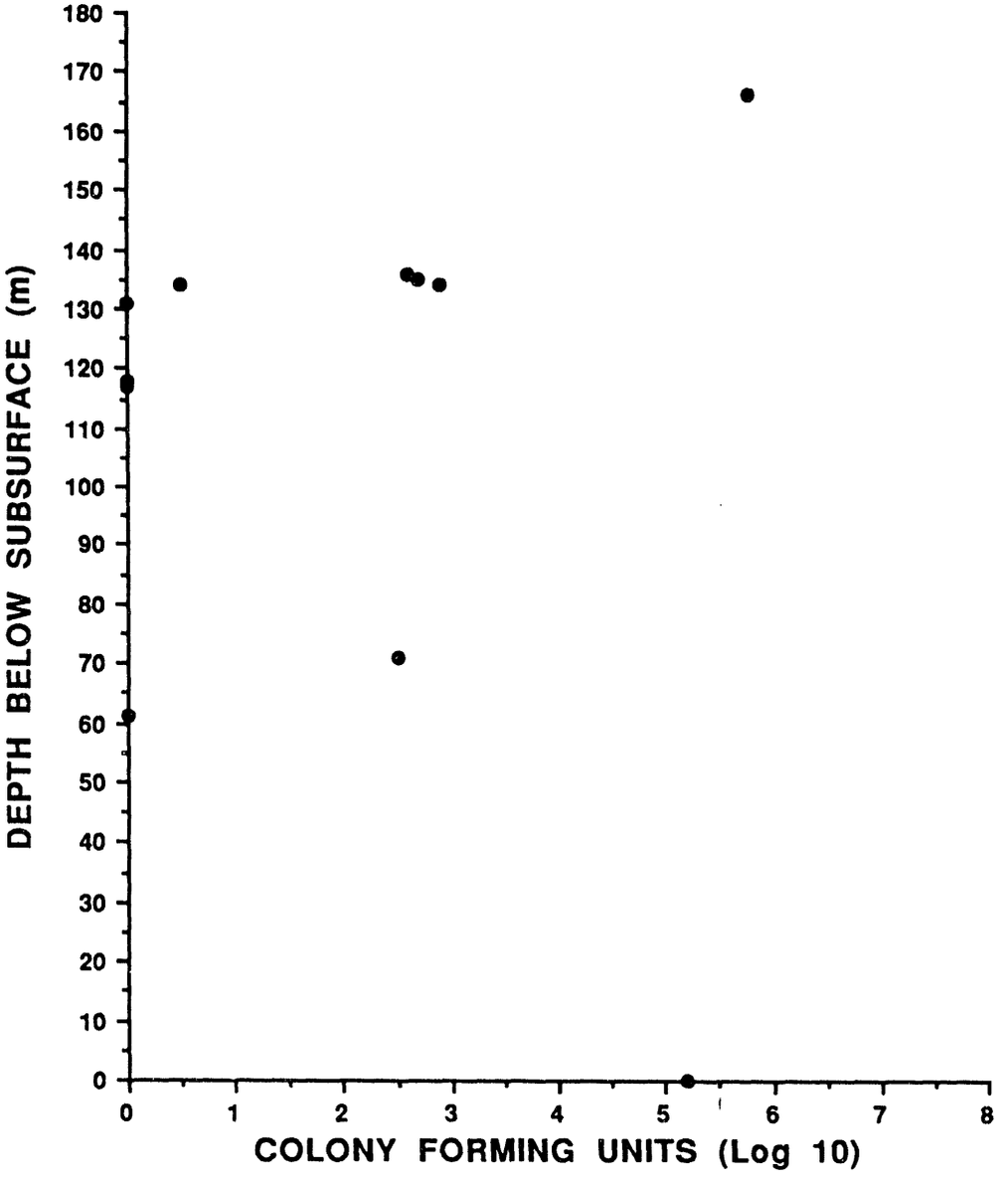
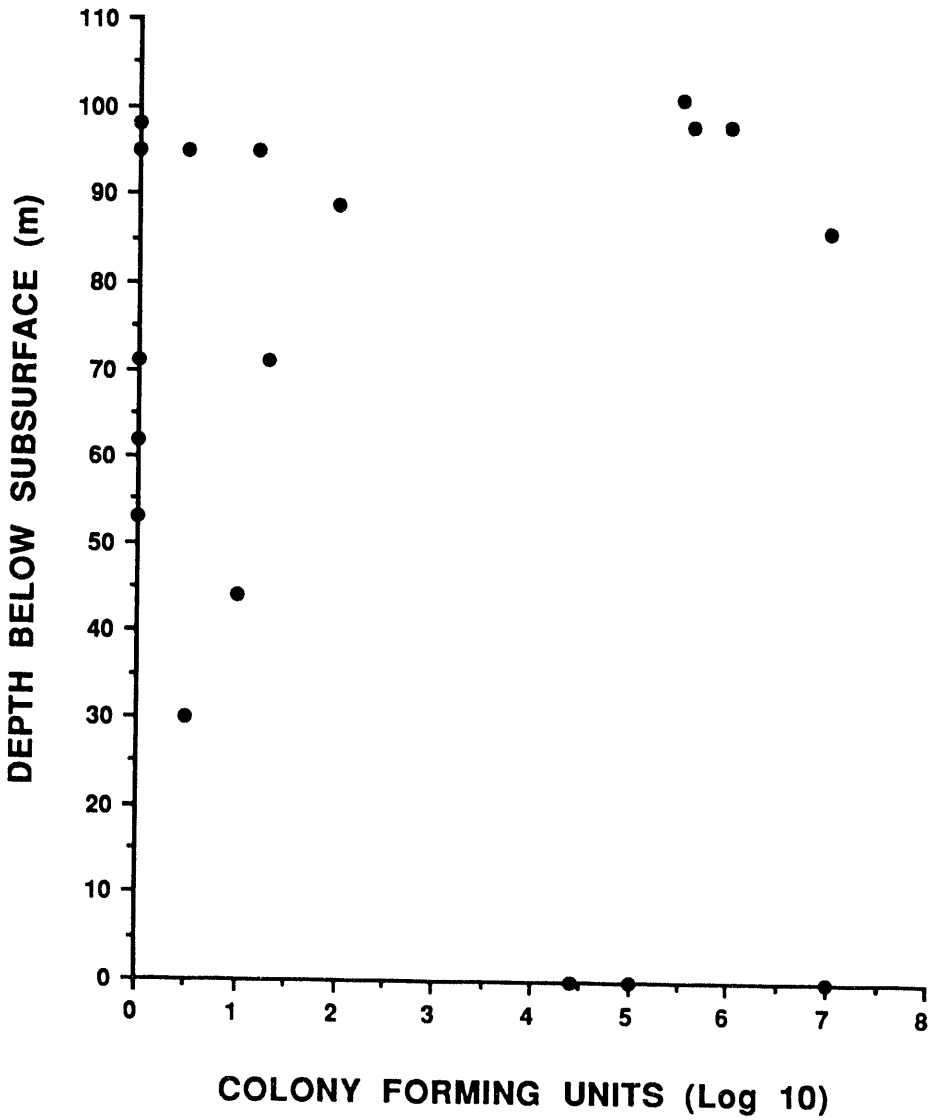


FIGURE 7. HR CFUs BY DEPTH



Tables 9 & 10. From our preliminary data we find no coorelation of sediment type or depth with numbers or types of bacteria found. The percentage of different trophic types for each sites are summarized in pie charts (Figure 8). The small number of culturable strains probably accounts for the lack of community structure. Graphs showing the relationship of trophic types to depth below the subsurface (Figures 9 & 10) show that obligate strains are generally associated with the deepest sediment samples at both sites.

- We initiated microelectrode experiments of selected samples to see if microbial activity is detectable with time. This approach apparently will not work given the low numbers of bacteria present in the core subsamples.

- We have transferred our facultative isolates to the DOE culture collection at Florida State University. Likewise, our obligate anaerobic cultures have been sent to Dr. D. Boone at the Oregon Graduate Institute.

- Presentations on this work:
  (i) Poster presentation by E.B. Sullivan, T.T. Hendrix, K.-T. Chung, and S.E. Stevens, "Isolation and characterization of anaerobic microorganisms from the deep subsurface." Third Annual Graduate Research Forum, Memphis State University, March 26, 1991.

TABLE 9. INEL: RELATIONSHIP OF SAMPLE GEOLOGY TO ANAEROBIC MICROBIAL GROWTH

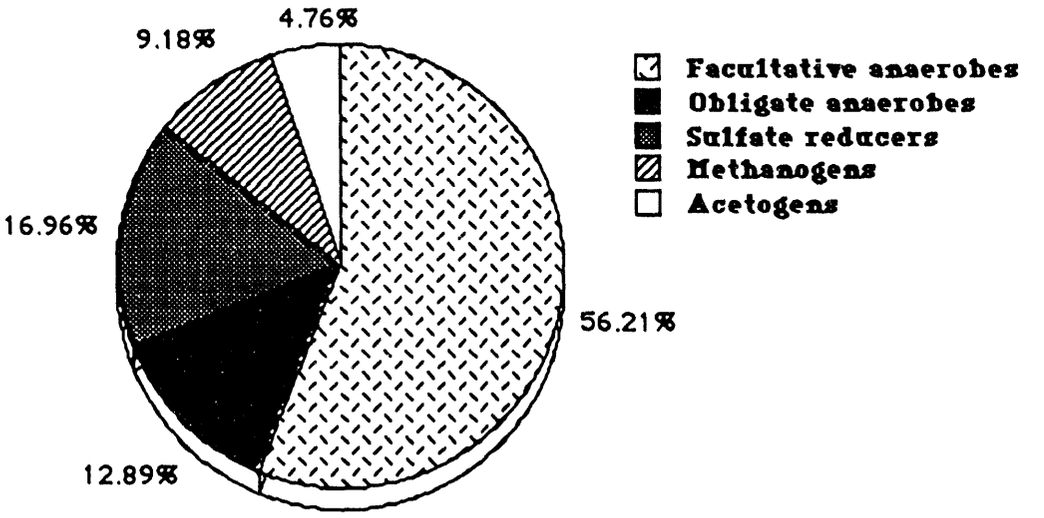
DOE Sample #	Depth (m)	Geology	100% PTYG
0-4-6	0	sand/surface	+
207	70.67	vesicular basalt	+
240/5	73.15	sediment/red sand	+
394/1-7	120.19	basalt	+
395/6-8	120.61	sediment (wet)	+
400/9-11	121.92	reddish sand	+
443/8-11	135.27	infill-sediment/rubble basalt	
448/0-3	136.55	crushed basalt	sterilized
water table			
451/10-452/10	137.86	basalt/sediment	+
452/1-453/1	137.95	sediment	
452/11-453/1	138.07	clay/sediment-basalt rubble	+
455/10-456/5	139.03	basalt/silt/clay	+
458/6-459	139.87	vesicular basalt	+
520/2	158.56	sediment	spiked
560-564	171.30	sand/interbed (wet sand)	+

TABLE 10. HR: RELATIONSHIP OF SAMPLE GEOLOGY TO ANAEROBIC MICROBIAL GROWTH

DOE Sample #	Depth (m)	Geology	100% PTYG
HMB-1	0	surface	+
0007A	0	surface	+
0007B	0	overlay ?	+
107	31.85	gravely muddy sand	+
207	47.03	sand	+
307	53.55	gravely sand	+
407	62.72	muddy sand	+
507	71.75	muddy sand ?	+
537	73.43	mud ?	+
607	89.18	muddy gravel	+
707	89.86	muddy gravel/agrillic soil (Fe)	+
807a	96.23	muddy sandy gravel	
907a	98.79	muddy gravel	
water table			
1007	99.85	muddy gravel (Fe)	+
1107	100.61	silty sand ?	
1407	101.29	gravelly sand	+
1207	101.62	gravel or sand ?	
1307	103.11	mud/sand/gravel ?	+

FIGURE 8.

PERCENTAGE OF TROPHIC TYPES - YAKIMA SAMPLES



PERCENTAGE OF TROPHIC TYPES - INEL SAMPLES

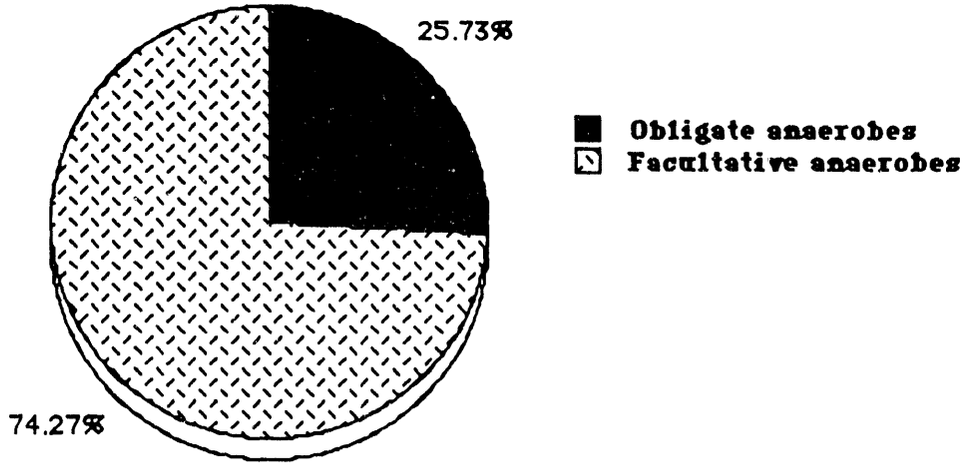


FIGURE 9. TROPHIC TYPES BY DEPTH FOR INEL SAMPLES

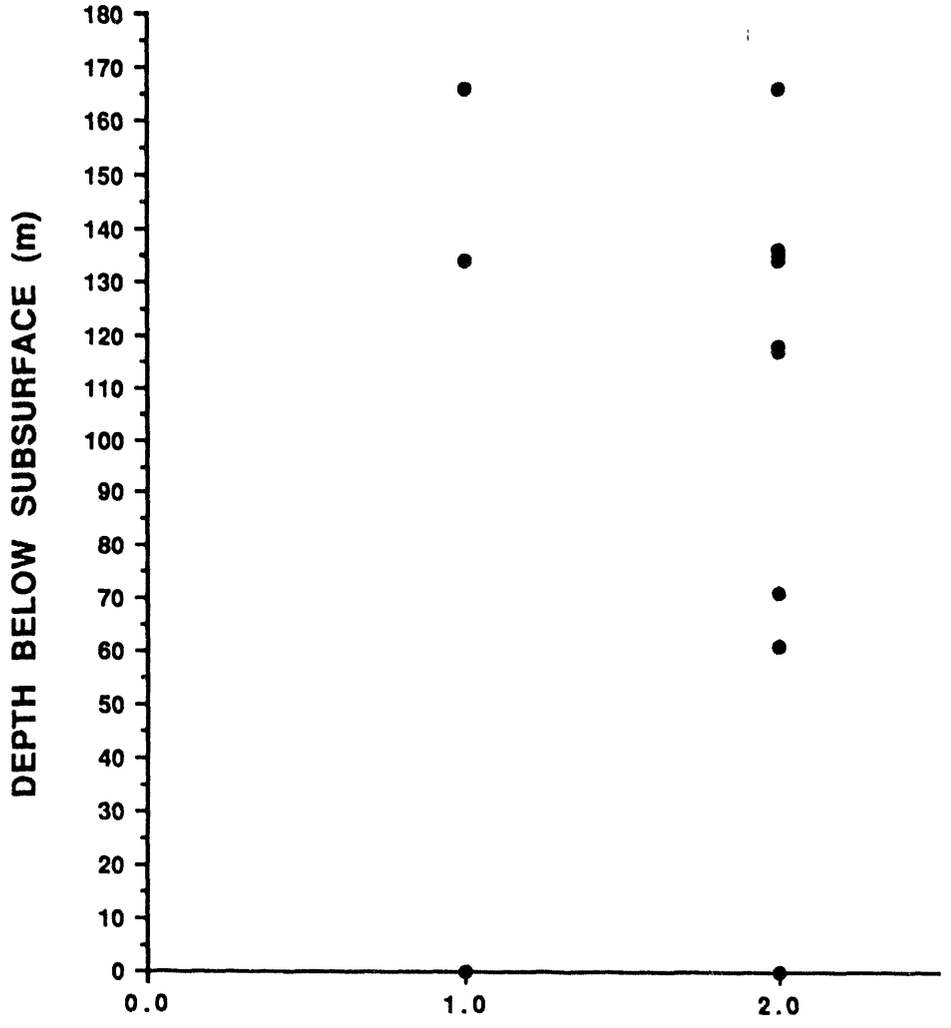
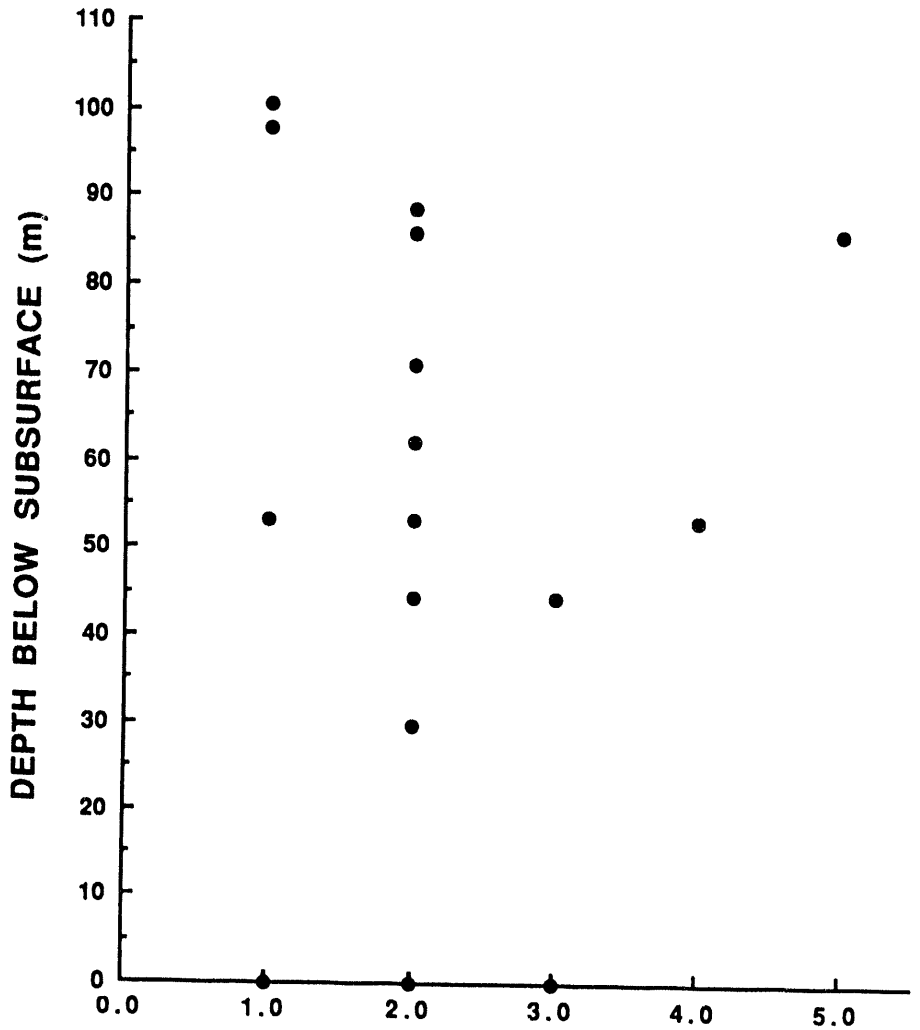


FIGURE 10. TROPHIC TYPES BY DEPTH FOR HR SAMPLES



TROPHIC TYPES: 1-Obligate, 2-Facultative,
3-Sulfate reducer, 4-Methanogen, 5-Acetogen

Facultatively and Obligatively Anaerobic Bacteria from the Deep Subsurface. M. ROTHSCHILD*, S.E. STEVENS, JR., K.-T. CHUNG, Memphis State Univ, Memphis, TN

A variety of different media were used to isolate facultatively (FAB) and obligately anaerobic bacteria (OAB). These bacteria were isolated from core subsamples obtained from boreholes at the Idaho Nat. Engineering Lab. (INEL) or at the Hanford Lab. (Yakima). Core material was sampled at various depths to 600 feet below the surface. All core samples with culturable bacteria contained at least FAB making this the most common physiological type of anaerobic bacteria present in the deep subsurface at these two sites. INEL core samples are characterized by isolates of both FAB and OAB. No isolates of acetogenic, methanogenic, or sulfate reducing bacteria were obtained. Yakima core samples are characterized by a marked predominance of FAB in comparison to OAB. In addition, isolates of acetogenic, methanogenic, and sulfate reducing bacteria were obtained. The Yakima site has the potential for complete anaerobic mineralization of organic compounds whereas this potential appears to be lacking at INEL.

Q-190 Facultatively and Obligatively Anaerobic Bacteria from the Deep Subsurface. M. ROTHSCHILD*, S.E. STEVENS, JR., K.-T. CHUNG, Memphis State Univ, Memphis, TN

A variety of different media were used to isolate facultatively (FAB) and obligately anaerobic bacteria (OAB). These bacteria were isolated from core subsamples obtained from boreholes at the Idaho Nat. Engineering Lab. (INEL) or at the Hanford Lab. (Yakima). Core material was sampled at various depths to 600 feet below the surface. All core samples with culturable bacteria contained at least FAB making this the most common physiological type of anaerobic bacteria present in the deep subsurface at these two sites. INEL core samples are characterized by isolates of both FAB and OAB. No isolates of acetogenic, methanogenic, or sulfate reducing bacteria were obtained. Yakima core samples are characterized by a marked predominance of FAB in comparison to OAB. In addition, isolates of acetogenic, methanogenic, and sulfate reducing bacteria were obtained. The Yakima site has the potential for complete anaerobic mineralization of organic compounds whereas this potential appears to be lacking at INEL.

Microbiolal Ecology of Deep Subsurface Anaerobic Bacteria from two Boreholes at the DOE Hanford Lab. M. ROTHSCHILD*, S. E. STEVENS, JR., Memphis State Univ, Memphis, TN 38152

A variety of different media and two reducing agents (cysteine-HCl, titanium citrate) were used to isolate anaerobic bacteria from subsamples provided from two boreholes located at the Hanford Lab. Both boreholes represent the main geological formations and geochemical conditions of an area designated as the Yakima Barricade. Morphological (cell and colony characteristics) and physiological traits of all culturable isolates were determined by standard microscopic and physiological tests. We used these traits to characterize the type(s) of anaerobes found at various subsurface locations. We were provided geological and geochemical data from companion subsamples that we used to determine ecological characteristics. Our results suggest that anaerobes that utilize different chemotrophic pathways are usually not found at the same depth nor are they associated with the same geological characteristics.

Q-398 Microbiolal Ecology of Deep Subsurface Anaerobic Bacteria from two Boreholes at the DOE Hanford Lab. M. ROTHSCHILD*, S. E. STEVENS, JR., Memphis State Univ, Memphis, TN 38152

A variety of different media and two reducing agents (cysteine-HCl, titanium citrate) were used to isolate anaerobic bacteria from subsamples provided from two boreholes located at the Hanford Lab. Both boreholes represent the main geological formations and geochemical conditions of an area designated as the Yakima Barricade. Morphological (cell and colony characteristics) and physiological traits of all culturable isolates were determined by standard microscopic and physiological tests. We used these traits to characterize the type(s) of anaerobes found at various subsurface locations. We were provided geological and geochemical data from companion subsamples that we used to determine ecological characteristics. Our results suggest that anaerobes that utilize different chemotrophic pathways are usually not found at the same depth nor are they associated with the same geological characteristics.

(ii) Poster presentation as above at the annual meeting of the Western Collegiate Division of the Tennessee Academy of Science, Rhodes College, April 13, 1991.

(iii) Rothschild, M., S. E. Stevens, Jr., and K.-T. Chung. "Facultatively and obligatively anaerobic bacteria from the deep subsurface." Abst. No. Q-190. Annual Meeting Amer. Soc. Microbiol., New Orleans, LA, p. 367 (1992).

(iv) Rothschild, M. and S. E. Stevens, Jr. "Microbial ecology of deep subsurface anaerobic bacteria from two boreholes at the DOE Hanford lab." Abst. No. Q-398. Annual Meeting Amer. Soc. Microbiol., Atlanta, GA, p. 419 (1993).

- Manuscripts in preparation or anticipated:
  (i). Rothschild, M., S. E. Stevens, Jr. and K.-T. Chung. Enumeration and characterization of deep subsurface anaerobic bacteria from two western DOE study sites. (manuscript appended, probably will be submitted to Appl. Environ. Micrcbiol.)

  (ii). Rothschild, M. and S. E. Stevens, Jr. Physiology of deep subsurface microorganisms in the absence of oxygen (anticipated).

(iii). Balkwill, D. L., M. Rothschild, and S. E. Stevens, Jr. Phospholipid profiles of anaerobic and facultatively anaerobic bacteria from the deep subsurface. (anticipated).

(iv). Rothschild, M., and S. E. Stevens, Jr. Phylogeny of anaerobic and facultatively anaerobic bacteria from the deep subsurface. (anticipated).

(v). Rothschild, M., and S. E. Stevens, Jr. Sulfate reducing bacteria from the deep subsurface. (anticipated).

Enumeration and Characterization of Deep Subsurface Anaerobic Bacteria from Two Arid Western Sites

Marjorie Rothschild, S. E. Stevens, Jr.*, and K.-T. Chung

Department of Biology

Memphis State University

Memphis, TN 38152

Running title: Deep Subsurface Anaerobic Bacteria

*Corresponding author

phone (901) 678-3705

FAX: 901-678-4457

ABSTRACT

Anaerobic bacteria were isolated from deep subsurface sediment samples taken at study sites in Idaho (INEL) and Washington (HR) by culturing on dilute and concentrated medium. Morphologically distinct colonies were purified, and their responses to 21 selected physiological tests were determined. Although the number of isolates was small (18 INEL, 27 HR) some general patterns could be determined. Most strains could utilize all the carbon sources, however the glycerol and melizitose utilization was positive for 50% or less of the HR isolates. Catalase activity (27.78% at INEL, 74.07% at HR) and tryptophan metabolism (11.12% at INEL, 40.74% at HR) were significantly different between the two study sites. MPN and viable counts indicate that sediments near the water table yield the greatest numbers of anaerobes. Deeper sediments also appear to be more selective with the greatest number of viable counts on low-nutrient mediums. Likewise, only strictly obligate anaerobes were found in the deepest sediment samples. Selective media indicated the presence of methanogens, acetogens, and sulfate reducers at only the HR site. Because there is no overlap in these trophic types we conclude that no community of anaerobes exists at either INEL or HR. The unique results from these two arid western sites also indicates that no general patterns can be formulated for deep subsurface microbial ecology at this time.

INTRODUCTION

Within the past ten years there has been an increased awareness of the potential of finding a variety of microorganisms in deep subsurface samples (12,16,30). We now realize that contaminants can move through the subsurface into aquifers where they would pose a threat to drinking supplies (8,29). Most of the study sites have been associated with aquifers since the presence of deep subsurface microbes may effect water supplies that were once considered to be immune from microbial effects. Deep subsurface microorganisms could also effect both the movement and degradation of environmental contaminants (2,4,9,10,14,18-25,27).

Because the Department of Energy (DOE) has several sites around the country that suffer from heavy contamination it began a deep subsurface microbial program to address a variety of questions. The first study sites were in southeast coastal plains associated with a number of aquifers and consisting of mainly sandy type formations that had a high potential for groundwater movement (2,5,13,23). It is therefore of interest to see what numbers and types of microorganisms are found in western areas where formations are less porous and there is less potential for groundwater flow.

The purpose of this study was to enumerate and characterize anaerobic bacteria from deep subsurface samples taken at two western DOE sites. We also were interested in the relationship between geological characters such as sample sediment type and

sediment type and sample depth, and the types of anaerobic bacteria found in those samples. In relating the microbial and geological results we hoped to gain a better picture of the subsurface ecology at these sites.

MATERIALS AND METHODS

Subsurface samples.

The subsurface samples examined in this study were obtained from two study sites operated by the Department of Energy as part of its Subsurface Microbiology Program. One series of samples represents a borehole at the Idaho National Engineering Laboratory (INEL) and the other series delineates a borehole in the Yakima Barricade of the Hanford Reservation, Washington (HR). Sediment cores from the boreholes were subdivided and sent to various researchers for specific types of analysis, geological, hydrological, chemical, biological. Therefore, the abiotic data presented here represents analysis done by co-project researchers. All of the samples were obtained and handled aseptically and shipped on ice by DOE scientists working at the sites. The samples were stored at $4^{o}C$ until they were examined as described below.

Total cell counts.

An acridine orange direct count (AODC) method was used to determine the total numbers of microbial cells in the subsurface sediments. The method used was that of Ghiorse and Balkwill (15,31). Cell counts were averaged over 25 randomly chosen

fields.

Viable cell counts.

Numbers of viable bacteria (CFU) in subsurface sediments were estimated by plate counts within 48 h after receipt of each sample. Plate counts were done by first creating a 1% subsurface sediment solution using sterile 0.1% sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$; pH 7). Soil samples were quickly weighed and placed in a sterile Waring blender, blended for 1 min (two 30 s bursts separated by a 30 s rest interval) to produce a well-dispersed suspension. The blended suspensions were quickly poured into sterile sample jars and sealed. Serial dilutions (10^{-2} to 10^{-5}) were then prepared utilizing an anaerobic phosphate buffer solution ($Na_2HPO_4 \cdot H_2O$; $NaH_2PO_4 \cdot H_2O$; NaCl; pH 7). The resulting dilutions were spread-plated in triplicate on each of four pre-reduced plating media: Egg yolk emulsion enriched (I), dilute Egg yolk (II), PTYG agar (III), dilute PTYG agar (IV).

Egg yolk emulsion medium contained the following ingredients per liter of distilled water: Na_2EDTA, 0.002 g; K_2HPO_4, 0.065 g; KH_2PO_4, 0.035 g; $NaCO_3$, 0.025 g; NaCl, 0.075 g; $MgSO_4 \cdot 7H_2O$, 0.075 g; trypticase 15 g; glucose 5 g; yeast extract 1 g; chicken egg yolks, 120 ml; A-5 metals mix (see Stevens et al., 1973), 1 ml; ferric ammonium citrate 0.012 g; agar 15 g. Medium II was a 1:20 dilution of the above medium. A soft agar overlay was used for both Medium I and II. The ingredients of the soft agar overlay were per liter: K_2HPO_4, 6.5 g; KH_2PO_4, 3.5 g; Na_2CO_3, 2.5 g; cysteine

$HCl \cdot H_2O$, 1 g; resazurin, 0.1 ml; agar, 7.5 g. Medium III contained the following ingredients per liter of distilled water: glucose, 10 g; yeast extract, 10 g; peptone, 5 g; tryptone, 5 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; $CaCl_2 \cdot 2H_2O$, 0.07 g; agar, 15 g. Medium IV was a 1:100 dilution of the first four ingredients of Medium III.

Plates were incubated anaerobically for three weeks, and then evaluated by counting the total number of colonies. A Friedman test (Statview 512+) was run to compare the number of viable counts per sample over the four types of medium. A comparison of the full strength and dilute media was made using a standard t-Test (Statview 512+). Isolates were transferred to 10 ml of broth medium that consisted of pre-reduced Medium III without agar.

Most probable number (MPN) determinations were made utilizing roll tubes containing 5 ml of pre-reduced Medium III with agar. The inoculum was 0.1 ml of either the 10^{-1} or 10^{-2} serial dilution from the sample. Sterile syringes were used to inject the dilution samples into stoppered vials containing still liquid medium. The vials were then rolled in a small layer of water to create a smooth coat of solid medium over the interior of the vials.

Aerobic counts.

Colony formation on 1% PTYG plates incubated aerobically was also noted.

Isolation and cultivation of subsurface bacteria.

The bacterial cultures examined in this study were isolated

from plates originally used for enumeration of viable colonies. Isolates were characterized by standard microbiological methods (Gerhardt, 1981). In addition, the API 20A system, Analytical Profile Index for Anaerobes (API Analytab Products) was used to characterize metabolic differences of colonies grown on plates and in broth cultures. Comparisons of the results from the two test sites were made using standard t-tests and correlations (Statview 512+).

Special medium for sulfate reducers was based on Postgate's formula (26): KH_2PO_4, 0.5 g; NH_4Cl, 1 g; Na_2SO_4, 1 g; $CaCl_2 \cdot 2H_2O$, 0.67 g; $MgCl_2 \cdot 7H_2C$, 2 g; sodium lactate (Sigma) 1 g; yeast extract 1 g; ascorbic acid 0.1 g; thioglycollic acid, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.5 g; agar, 15 g; pH 7.6. A liquid medium (Medium B in ref. 26) was also used to check for the presence of sulfate reduction.

Special media for methanogens and acetogens were designated as H_2-CO_2 medium or methylamine-acetate medium, respectively. The H_2-CO_2 medium contained in 900 ml of deionized water NH_4Cl, 1 g; $K_2HPO_4 \cdot 3H_2O$, 0.4g; $MgCl_2 \cdot 6H_2O$, 1.0g; $CaCl_2 \cdot 2H_2O$, 0.4 g; Na_2-EDTA$\cdot 2H_2O$, 1 ml (500 mg/ml); 1 ml of resazurin (0.1%) and 1 ml of trace mineral solution. The trace mineral solution contained per 100 ml of distilled water: $CoCl_2 \cdot 6H_2O$, 150 mg; $MnCl_2 \cdot 4H_2O$, 100 mg; $FeSO_4 \cdot 7H_2O$, 100 mg; $ZnCl_2$, 100 mg; $AlCl_3 \cdot 6H_2O$, 40 mg; $Na_2WO_4 \cdot 2H_2O$, 30 mg, $CuCl_2 \cdot 2H_2O$, 20 mg; $NiSO_4 \cdot 6H20$, 20 mg; H_2SeO_3, 10 mg; H_3BO_3, 10 mg, $NaMoO_4 \cdot 2H_2O$, 10 mg. The pH of the

trace mineral solution was adjusted to 2.5-3.0 with 1N HCl and stored at 4°C.

The mineral salts and trace metal solution was heated to boiling and 1.0 g of yeast extract (Difco) and 1.0 g of trypticase peptone (BBL) were added and the solution brought back to a boil. The hot medium was then bubbled with O_2-free CO_2 until it was cool. The CO_2 gas stream was passed through a tangled mass of hot copper wire which removed the O_2. After cooling, $NAHCO_3$ (8.6 g) and mercaptoethane -sulfonic acid (0.5 g) were added (under the gentle flow of O_2-free gas). The medium was then dispensed (8.9 ml) anaerobicalluy under the gentle flow of an 80% H_2/20% CO_2 gas mixture into VPI anaerobe tubes that were sealed with #1 butyl rubber stoppers and autoclaved.

The methylamine-acetate medium was prepared using the same mineral salts and trace metal solution described above. Upon cooling mercaptoethanesulfonic acid (0.5 g), methylamine (1.92 g) and Na-acetate (4.1 g) were added under the gentle flow of O_2-free nitrogen gas. The medium was dispensed as previously described under a flow of O_2-free nitrogen gas.

Both autoclaved media were stored at room temperature. Before use, 0.1 ml of 2.5% sterile cysteine-HCl solution was injected into each tube by syringe through the rubber stopper while flushing with sterile O_2-free nitrogen gas. The 2.5% cysteine-HCl was also prepared under a gentle flow of O_2free nitrogen gas. The inoculum for these media were 1 ml of the anaerobic PBS 10^{-2} sample dilutions. Samples were incubated

at room temperature for two months. Growth and gas uptake in the H_2-CO_2 medium were taken as indicative of the presence of methanogens, and no growth or gas uptake coupled with growth in the methylamine-acetate medium was taken as indicative of the presence of acetogens.

RESULTS

Total cell counts (AODCs).

The total cell count (AODC) of bacteria in all but a couple of the sediments sampled were very low ranging from 0 to 10 total cells over 25 fields counted. Therefore, no significant conclusions can be made based on these results. One sample (207, 61 m) from INEL and one sample (607, 86 m) from HR were determined to have over 100,000 cells per ml of sample.

Viable cell counts.

Viable cell (plate) counts for both series of samples ranged from no growth to counts of 10^7 CFU g (dry wt)$^{-1}$. The fluctuations in viable counts for each sample site did not indicate any association with a particular soil composition. Results for both sites are recorded in Table 1.

There were no significant differences ($p = 0.318$) in viable counts/per sample over the four types of medium for the INEL samples. Also t-Tests comparing the full strength and dilute medium produced no significant differences. The HR site had more viable counts per sample and a comparison of medium types over samples was significant ($p = 0.003$). Like the INEL

results, the t-Test comparison of Medium I and Medium II was not significant, but viable counts on Medium III and IV ($p = 0.014$) did differ. For both sites, the least number of colonies developed on Medium I, and the highest number developed on Medium IV.

The MPN results from the roll tube cultures were similar at both sites. In both cases, only samples taken within 5 m of the water table, or from the surface had viable numbers. There was also no significant correlation between depth and viable counts on the plates incubated aerobically, though the greater depths at both sites (below 120 m INEL, below 85 m HR) produced samples with the highest aerobic cell counts excluding surface samples.

Colony and cell morphologies on anaerobically incubated plates.

The predominant colony and cell morphologies for both series of samples are presented in Table 2. Both sample series contained mostly rods or coccobaccillus (cb) type cells singly or in pairs. For both sites, there was an association between the following: Gram + cell type and spindle shaped, small, white, opaque colony form. In all but two samples from HR, this association was of paired cb. No particular cell shape was associated with this pattern for the INEL site. However, Gram -, circular shaped, large, white, transparent colonies made up another major association of cultured INEL forms. Spindle colonies are indicative of obligate anaerobes and were located below the surface on culture plates and within the solidified medium in roll tube cultures. This colony type was associated

with only Gram + bacteria at both sites.

Gram + cb forms dominated at each site (Table 2). Approximately 53% of the cb cells cultured from INEL samples and 25 % of the cb cells cultured from HR samples formed spindle shaped colonies. Other major differences were in the percentage of Gram - cb (6% at INEL & 20% at HR), and the percentage of Gram + rods (17% at INEL & 6% at HR).

Physiological characteristics.

A comparison of metabolic abilities on anaerobically incubated medium in relation to depth is presented in Fig. 1. For both sites, the obligate forms were cultured from the deepest sediment samples. There is more metabolic diversity at the HR site, but metabolic types do not seem to be associated with one another, with depth, or with specific geologies.

API results reveal few trends because most of the strains from both sites were capable of utilizing all the sugars (Table 3). However, a few comparisons can be made based on the other metabolic activities. Approximately the same percent of strains from both sites were positive for esculin hydrolysis and gelatin liquefaction. The predominant type of metabolic activity for all strains was esculin hydrolysis, and approximately 50% of the INEL strains and 25% of the HR strains only tested positive for this non-sugar type of metabolism. More diversity in metabolism was shown in the HR strains, mainly because, a much larger percent of the strains were positive for both catalase activity and tryptophan metabolism. About 6% of the INEL strains were positive for tryptophan metabolism, but not

catalase activity; none of the HR strains showed this result. The correlation between the results from the two sites for these traits was 0.58 with an r^2 value of 0.336, n.s.

For INEL samples, all spindle colony strains were positive for all sugars. If a strain did not assimilate rhamnose then it also did not assimilate raffinose. Six INEL strains were not able to utilize raffinose, while only one strain, 407B, from HR was not able to assimilate this sugar. HR samples had negative sugar results when cultured on solid medium. Unique results were associated with only a few strains at either site. Sugars that were not assimilated by at least one strain include the following: arabinose, mannose, sorbitol, and cellobiose.

Other trends from HR isolates include an association between a positive tryptophan metabolic response, positive catalase response, and a lack of glycerol utilization as a carbohydrate source. Also, when results indicated a lack of esculin hydrolysis, then sugar utilization patterns showed a lack of melezitose assimilation and usually a lack of assimilation of salicin and glycerol. No association between cell type, or sample depth was noted. A correlation of sugar metabolism results for both sites was only 0.44 with an r^2 value of 0.195, n.s.

Sulfate reduction was only noted in samples from HR. Surface samples HMB-1, 0007a, 0007b and sample 207 all showed evidence of sulfate reduction in both the liquid medium and in solid agar tubes. Evidence of a methanogen from sample 307 (HR) and an acetogen from sample 607 (HR) was also noted.

DISCUSSION

Isolatable anaerobic bacteria are present in the deep subsurface sediments at two western sites, INEL and HR. The low numbers seem to be consistent with other studies, including those from coastal plain sediments (19,23). Both our aerobic CFU results and other studies concerned with the aerobic microorganisms from these sites (1) have produced much lower numbers than those associated with the more porous coastal plain sediments (2,5,13,23). This may indicate that these types of sediments are less capable of maintaining any type of microbial life (14,17,21,28).

Though the overall growth for all samples was generally poor as seen in the CFU results, the most growth was seen at lower nutrient levels. Most of the samples contained anaerobic bacteria capable of utilizing more enriched medium; a result that agrees with Balkwill's (1989) suggestion that deep subsurface microorganisms must be versatile in meeting nutritional needs.

Anaerobic plate counts and MPN results do not reveal any association with sediment type at either study site, but do indicate that sediments that are less porous or further from a water source (surface water, water table) are less likely to contain viable anaerobes. The low plate counts probably account for the lack of differences between medium types for INEL samples. The HR site seems to contain more versatile anaerobes, and our results at this site support the hypothesis

that more growth will be obtained on lower nutrient medium.

In this study we found that Gram + coccoid or cb types predominated at each site. This seems to be consistent with a number of studies in Oklahoma (6,7). Those studies were also characterizing bacteria from less transmissive sediments. Studies characterizing bacteria from more porous type sediments have shown Gram - rods as the predominant type (2,20,28).

Obligate types for both sites were associated with the deepest samples probably because it is these samples that would be most likely to remain anaerobic over time. Bone and Balkwill (1989) suggest that deeper samples contain more selective bacteria, and that these types do best when grown on low nutrient levels. Our results showing strict anaerobes from the deepest sediments supports such a conclusion.

Our results reveal noticeable differences in the anaerobic bacteria associated with these two study sites. The HR site has both a greater number of viable bacteria and a greater diversity in culturable isolates. The colony and cell type percentages indicate that different assemblages of anaerobes are found at these sites. Furthermore, different metabolic constraints seem to be operating at these two sites as shown by both the API tests and selective medium studies. These results suggest that we must consider each study site as unique and governed by unique ecological constraints.

In his paper on community structure Drake (1990) defines a community as an "ensemble of species in some area whose limits are determined by the practical extent of energy flow." Though

there is more diversity in trophic types at the HR site, there is minimal overlap. We therefore suggest that the anaerobic bacteria from either site can not be considered as a community. Whether these anaerobes could be part of some subsurface microbial community spread over a wide volume of the subsurface can not be determined at present.

ACKNOWLEDGEMENTS

We thank Tracy Hendricks and Elizabeth Sullivan who helped with the initial culturing and plate counts. This work was supported by a grant from the U.S. Department of Energy (No. DE-FG05-90ER60991) and funds from the W. Harry Feinstone Chair of Excellence in Molecular Biology (SES).

LITERATURE CITED

1. _____. 1992. Deep microbiology program status, scribe notes. DOE Deep Microbiology Meeting, Las Vegas, NV, Jan 14-16.

2. **Balkwill, D. L.** 1989. Numbers, diversity, and morphological characteristics of aerobic, chemotrophic bacteria in deep subsurface sediments from a site in South Carolina. Geomicrobiol. 7:33-52.

3. **Balkwill, D. L.** 1990. Deep aquifer microorgansims. In, Isolation of Biotechnological Organisims from Nature, pp. 183-211. McGraw-Hill Publ. Co., New York.

4. **Balkwill, D. L. and W. C. Ghiorse.** 1985. Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma. Appl. Environ. Microbiol. **50**:580-588.

5. **Balkwill, D. L., J. K. Fredrickson, and J. M. Thomas.** 1989. Vertical and horizontal variations in the physiological diversity of the aerobic chemoheterotrophic bacterial microflora in deep Southeast Coastal Plain subsurface sediments. Appl. Environ. Microbiol. **55**:1058-1065.

6. **Beloin, R. M., J. L. Sinclair, and W. C. Ghiorse.** 1988. Distribution and activity of microorganisms in subsurface sediments of a pristine study site in Oklahoma. Microbiol. Ecol. **16**:85-97.

7. **Bone, T. L., and D. L. Balkwill.** 1988. Morphological and cultural comparison of microorganisms in surface soil and subsurface sediments at a pristine study site in Oklahoma. Microbiol. Ecol. **16**:49-64.

8. **Chapelle, F. H.** 1993. Ground-Water Microbiology & Geochemistry. John Wiley & Sons, Inc. New York. pp 424.

9. **Chapelle, F. H. and D. H. Lovley.** 1990. Rates of bacterial metabolism in deep coastal plain aquifers. Appl. Environ. Microbiol. **56**:1865-1874.

10. **Chapelle, F. H., J. T. Morris, P. B. McMahon, and J. L. Zelibor, Jr.** 1988. Bacterial metabolism and the $d^{13}C$ composition of ground water, Floridan Aquifer System, South Carolina. Geol. **18**:117-121.

11. **Drake, J. A.** 1990. Communities as assembled structures: do rules govern pattern? Tree **5**:159-164.

12. **Fliermans, C. B. and D. L. Balkwill.** 1989. Microbial life in deep terrestrial subsurfaces. BioSci. **39**:370-377.

13. **Fredrickson, J. K., D. L. Balkwill, J. M. Zachara, S. W. Li, F. J. Brickman, and M. A. Simmons.** 1991. Physiological diversity and distribution of heterotrophic bacteria in deep Cretaceous sediments of the Atlantic Coastal Plain. Appl. Environ. Microbiol. **57**:402-411.

14. **Fredrickson, J. K., T. R. Garland, R. J. Hicks, J.M. Thomas, S. W. Li, and S. M. McFadden.** 1989. Lithotrophic and heterotrophic bacteria in deep subsurface sediments and their relation to sediment properties. Geomicrobiol. **7**:53-66.

15. **Ghiorse, W. C. and D. L. Balkwill.** 1983. Enumeration and characterization of bacteria indigenous to subsurface environments. Dev. Ind. Microbiol. **24**:213-224.

16. **Ghiorse, W. C., and J. T. Wilson.** 1988. Microbial ecology of the terrestrial subsurface. Adv. Appl. Microbiol. **33**:107-172.

17. **Hazen, T. C., L. Jiménez, G. L. de Victoria, and C. B. Fliermans.** 1991. Comparison of bacteria from deep subsurface sediment and adjacent groundwater. Microbial Ecol. **22**:293-304.

18. **Hicks, R. J. and J. K. Fredrickson.** 1989. Aerobic metabolic potential of microbial populations indigenous to deep subsurface environments. Geomicrobiol. **7**:67-78.

19. **Jones, R. E., R. E. Beeman, and J. M. Suflita.** 1989. Anaerobic metabolic processes in deep terrestrial subsurface. Geomicrobiol. **7**:117-130.

20. **Kölbel-Boelke, J., E. Anders, and A. Nehrkron.** 1988. Microbial communities in the saturated groundwater environment II: Diversity of bacterial communities in a Pleistocene sand aquifer and their in vitro activities. Microbiol. Ecol. **16**:31-48.

21. **Konopka, A. and R. Turco.** 1991. Biodegradation of organic compounds in vadose zone and aquifer sediments. Appl. Environ. Microbiol. **57**:2260-2268.

22. **Lovley, D. H., F. H. Chapelle, and J. P. Phillips.** 1990. Fe(lll)-reducing bacteria in deeply buried sediments of the Atlantic coastal plain. Geol. **18**:954-957.

23. **Madsen, E. L. and J. M. Bollag.** 1989. Aerobic and anaerobic microbial activity in deep subsurface sediments from the Savannah River Plant. Geomicrobiol. **7**:93-101.

24. **McMahon, P. B. and F. H. Chapelle.** 1991. Microbial production of organic acids in aquitard sediments and its role in aquifer geochemistry. Nature **349**:233-235.

25. **Phelps, T. J., E. G. Raione, D. C. White, and C. B. Fliermans.** 1989. Microbial activities in deep subsurface environments. Geomicrobiol. **7**:79-92.

26. **Postgate, J. R.** 1984. The Sulphate-Reducing Bacteria, 2nd. ed., Cambridge University Press, New York. 208 pp.

27. **Sinclair, J. L., and W. C. Ghiorse.** 1989. Distribution of aerobic bacteria, protozoa, algae and fungi in deep subsurface sediments. Geomicrobiol. **7**:15-32.

28. **Sinclair, J. L., S. J. Randtke, J. E. Denne, L. R. Hathaway, and W. C. Ghiorse.** 1990. Survey of microbial populations in buried-valley aquifer sediments from northeastern Kansas. Ground Water **28**:369-377.

29. **Sulfita, J. M.** 1989. Microbiological principles influencing the biorestoration of aquifers. In, Transport and Fate of Contaminants in the Subsurface, EPA 625/4-89/019, pp. 85-99. Robert S. Kerr Environmental Research Laboratory.

30. **White, D. C., G. A. Smith, M. J. Gehron, J. H. Parker, R. H. Findlay, R. F. Martz, and H. L. Fredrickson.** 1983. The groundwater aquifer microbiota: biomass, community structure, and nutritional status. Dev. Ind. Microbiol. **24**:201-211.

31. **Wilson, J. T., J. F. McNabb, D. L. Balkwill, and W. C. Ghiorse.** 1983. Enumeration and characterization of bacteria indigenous to a shallow water-table aquifer. Ground Water **21**:134-142.

TABLE 1. Anaerobic plate counts.

DOE Sample number	MEDIUM I	MEDIUM II	MEDIUM III	MEDIUM IV	Average DEPTH (M)	SEDIMENT TYPE
			INEL samples			
0-4-6	NS	NS	NS	NS	0	sand/surface
207	NS	0	NS	NS	61	vesicular basalt
240/5	NS	NS	NS	NS	71	sediment/red sand
394/1-7	NS	NS	NS	NS	117	basalt
395/6-8	NS	NS	NS	NS	117	sediemnt (wet)
400/9-11	NS	0	NS	0	118	reddish sand
443/8-11	0	NS	NS	NS	131	infill sediment/rubble basalt
448/0-3	NS	NS	NS	NS	133	crushed basalt (sterilized)
451/10-452/10	0	0	NS	0	134	basalt/sediment[1]
452/1-453/1	$7x10^4$	NS	NS	NS	134	sediment
452/11-453/1	NS	0	NS	0	134	clay/sediment-basalt rubble
455/10-456/5	NS	NS	0	NS	135	basalt/silt/clay
458/6-459	NS	NS	NS	NS	136	vesicular basalt
520/2	0	NS	NS	NS	154	sediment (spiked)
560-564	NS	NS	NS	NS	166	sand/interbed (wet)
			HR Samples			
HMB-1	NS	NS	NS	4433	0	surface
0007A	0	$1.2x10^4$	NS	$1.7x10^4$	0	surface
0007B	0	NS	0	$3x10^4$	0	overlay
107	NS	NS	NS	NS	30	gravely muddy sand
207	0	NS	0	$7.4x10^5$	44	sand
307	NS	NS	NS	NS	53	gravely sand
407	0	0	0	0	62	muddy sand
507	0	0	0	0	71	muddy sand
537	0	0	0	0	71	mud
607	$1.3x10^4$	$7.2x10^4$	$9.6x10^4$	$2.1x10^4$	86	muddy gravel
707	0	NS	NS	340	89	muddy gravel/agrillic soil (Fe)
807	0	0	0	0	95	muddy sandy gravel
907	0	0	0	0	95	muddy gravel
1007	0	0	NS	NS	98	muddy gravel (Fe)[1]
1107	$4.8x10^4$	0	0	0	98	silty sand
1407	0	$1.2x10^5$	NS	$7x10^4$	98	gravely sand
1207	$3.4x10^5$	$3.8x10^5$	$1.9x10^5$	$4.4x10^5$	98	gravel or sand
1307	0	$>10^4$	NS	NS	101	mud/sand/gravel

[1]First sample below water table.

TABLE 2. Percentage of morphological types of bacteria.

COLONY TYPE	CELL TYPE BY GRAM TYPE				SITE
	CB+	CB-	ROD+	ROD-	
Spindle	39	0	6	0	INEL
Circular	28	6	11	11	
Spindle	20	0	3	0	HR
Circular	40	20	3	13	

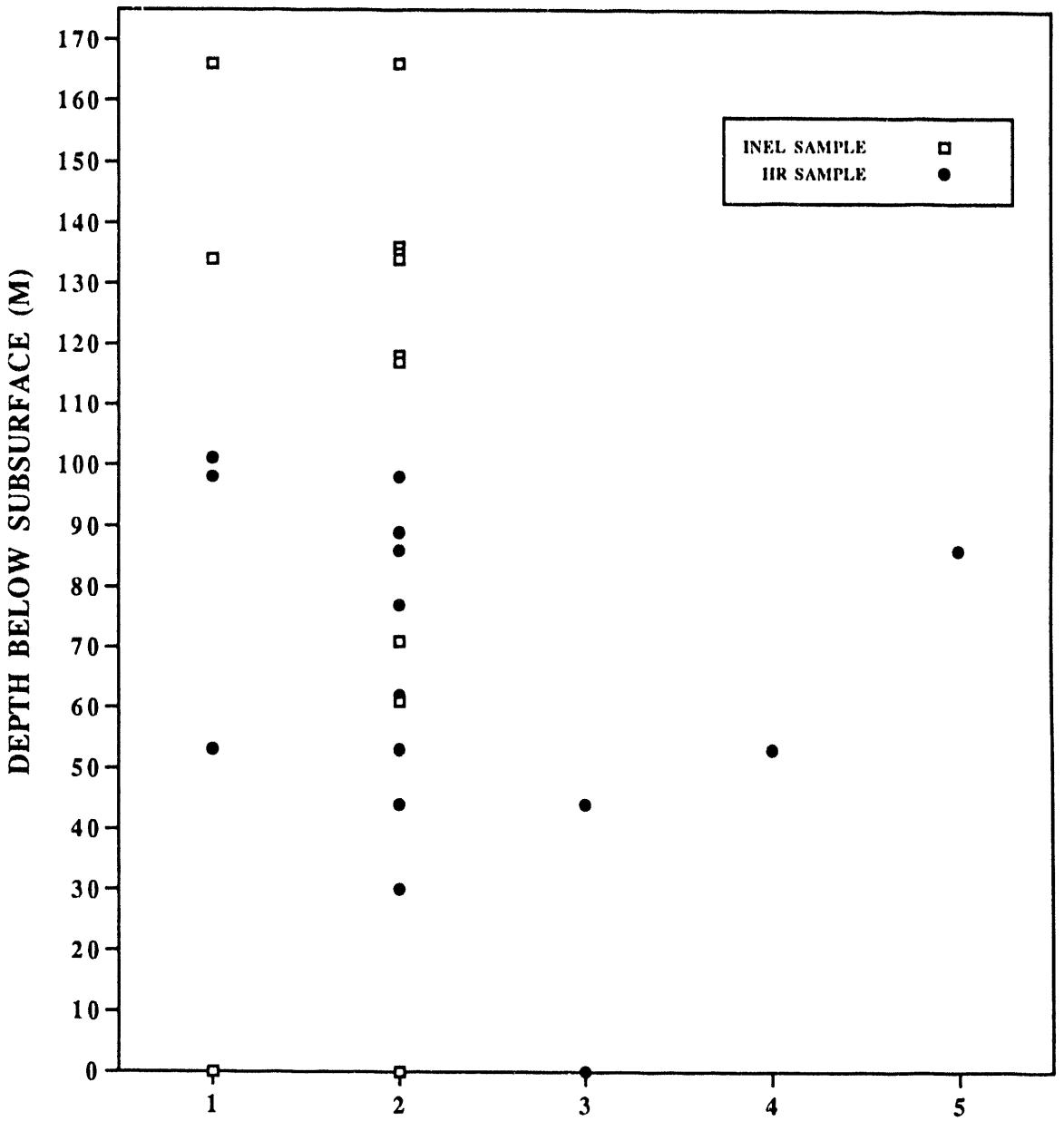
TABLE 3. Percentage of strains positive for physiological tests.

METABOLIC TRAITS	INEL (n=18)	HR (n=27)
Esculin hydrolysis	83.33	74.07
Only esculin positive[1]	55.56	25.93
Catalase activity	27.78	74.07
Tryptophane metabolism	11.12	40.74
Catalase & Tryptophane[2]	5.56	40.74
Gelatin liquefaction	5.56	7.41
Urease activity	0	3.70
ANAEROBIC ASSIMILATION OF:		
Glucose	100	100
Mannitol	100	100
Lactose	100	100
Saccharose (Sucrose)	94.44	100
Maltose	94.44	70.37
Salicin	94.44	70.37
d + Xylose	94.44	88.89
l + Arabinose	88.89	100
Glycerol	83.33	37.04
Cellobiose	100	96.30
Mannose	94.44	100
Melezitose	83.33	51.85
Raffinose	72.22	96.30
Sorbitol	94.44	100
Rhamnose	83.33	100
Trehalose	100	100

[1]Esculin was positive, but there was no catalase or tryptophan activity.
[2]Both catalase and tryptophane activity were positive.

FIGURE LEGENDS

Figure 1. Trophic types of bacteria by depth at INEL and HR.



TROPHIC TYPES: 1-Obligate, 2-Facultative,
3-Sulfate reducer, 4-Methanogen, 5-Acetogen

END

DATE
FILMED
2/11/94