WRC RESEARCH REPORT NO. 57

MICROBIAL MODIFICATION OF GROUND WATER

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FINAL REPORT

Project No. A-038-ILL

The work upon which this publication is based was supported by funds provided by the U. S. Department of the Interior.

UNIVERSITY OF ILLINOIS
WATER RESOURCES CENTER
2535 Civil Engineering Building
Urbana, Illinois 61801
August, 1972

ABSTRACT

MICROBIAL MODIFICATION OF GROUND WATER

When ground water is tapped by wells, microbial and chemical deposits often develop. Sloughing and clogging may occur in the distribution system adding considerable expense to the operation of the water systems as well as imparting taste and odor to the water itself. The purpose of this project has been to define the physical and microbial basis of these deposits using microbial flocs found in Southern Illinois as a "model system." These flocs proliferate at the air-water interface of a domestic flush tank producing copious amounts of flocculent material. Observation of the flocs by phase microscopy revealed a dense population of bacteria with several distinct morphological types. Analysis by scanning and transmission electron microscopy revealed that the floc members reside in a matrix and that the consortium consists of two ultrastructurally distinct types of bacteria. Results of chemical analysis of the well water indicated low levels of organic material, whereas results of gas chromatographic analysis indicated high amounts of methane to be present in the water. The predominant organism, an elipsoidal rod, was isolated from floc enrichments grown under a methane-air atmosphere. Two organisms of a second morphological cell type have also been isolated and their unique nutritional properties investigated. Extracellular matrix produced by the two organisms appear to be responsible for the formation of the floc. A number of heterotrophic organisms have also been isolated from the consortium. Cross-feeding experiments involving mixed cultures of the consortium isolates revealed a microbial food chain to exist with methane as the primary energy source for the development of these aquatic consortia. Dissolved methane in ground waters is a previously unappreciated energy source for the development of microbial communities in water supplies.

Wolfe, R. S.
MICROBIAL MODIFICATION OF GROUND WATER
University of Illinois Water Resources Center Report No. 57.
KEYWORDS--microbial floc/methane in ground water

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INTRODUCTION

This report covers investigations concerning the activities of microorganisms involved in the modification of ground water. In many areas of Illinois the value of ground water has been affected.

In exploitation of these resources microbial and/or chemical depositions add considerable expense to the operation of wells, water systems and subsequent use of the water. These problems never get better but get worse with continued operation. The distribution systems become fouled, clogging and head loss result. Taste and odor problems increase as the microbial food chain and its involvement become more complex. Sloughing occurs with nuisance and aesthetic problems being added to the expense of maintenance of the treatment and distribution system. An attempt has been made to define the basis of such fouling and to determine the relationships leading to such development.

It is believed that study of a "model system" representative of those seen in the state will contribute to an understanding of these factors. The "model" consortium dealt with in this study develops naturally in flush tanks of the State National History Survey laboratory near Sullivan, Illinois. The purpose of this project has been to define the physical and microbial basis of flocculent deposition when ground water is tapped by wells.

MATERIALS AND METHODS

Chemical analysis of well water

Water samples from the Sullivan sample site were analyzed for dissolved minerals. All determinations complied to Standard Methods for the Examination of Water and Waste Water (1). Analysis was provided by the Illinois State Water Survey Laboratories.

Water temperature was recorded in the field; pH was recorded upon return to the laboratory by using a pH meter (model 76, Beckman Instruments).

Sampling procedures

Water samples were collected from the water system in 1 gallon narrow mouth screw cap polyethylene bottles. The bottles were filled to overflowing from the tap and then capped tightly.

Floc samples were picked from the flush tanks with sterile spatulas and placed in sterile rubber stoppered test tubes or sterile 250 ml centrifuge bottles.

Gas samples were collected by directing a stream of water from the tap into an inverted flask filled with water which was displaced by the released gas. The water flow was 3 gallons per minute. Upon collection of gas, the flask was tightly capped with a metal screw lid.

Floc and water samples were transported to the laboratory on ice. Inoculation of enrichments and plates were completed within four hours of sample collection.

Gas analysis of well gas

Sample collection and analysis was performed by the Illinois State Geological Survey Laboratories. A Perkin-Elmer model 154 L gas chromatograph was used. O_2, N_2 and CH_4 levels were determined on a molecular seive

5a column at 25 C. Other gasses were determined on a silicone oil 200/500 coated five brick column at 90 C.

Light Microscopy

A Carl Zeiss Photomicroscope was employed for all phase-contrast observations.

Electron Microscopy

For thin sectioning, a fixation procedure was modified from that described by Kellenberger, et al. (5). Equal amounts of a liquid culture or floc and a solution of 2% osmic acid in acetate-veronal buffer which contained 0.1M $CaCl_2$ at pH 6.1 were mixed at room temperature. After 15 min the cells were centrifuged and suspended in 1% osmic acid in the same buffer. Following incubation over night at room temperature, the cells were centrifuged and suspended in 1.5% agar at 50 C. The agar-cell suspension was cooled and cut into cubes. The agar blocks were placed for 2 hr in 0.5% uranyl acetate in the same acetate-veronal buffer.

The agar cubes were dehydrated through a graded series of water-ethanol mixtures and then were placed in propylene oxide. They were infiltrated with a propylene oxide-Epon mixture and finally embedded in Epon by standard methods (8). Sections were cut with a diamond knife on a Reichert OMU-2 ultramicrotome. The sections were stained by lead citrate, as described by Reynolds (11) and were examined in an RCA EMU-3D electron microscope.

Scanning electron microscopy

Floc samples were fixed as previously described, freeze dried and coated with gold paladium by standard methods. Specimens were examined in a Cambridge model E 25 scanning electron microscope.

Media

Medium PPYE contained protease peptone and yeast extract at 0.3% (w/v) each prepared in tap water. Media WS and WSY contained the basal salts as described by Whittenbury (12) with the trace mineral solution described by Wolfe (13) being used. The WSY medium contained an additional 0.05% (w/v) yeast extract. Medium FDM contained mineral salts and buffer as described by Foster (4) in addition to 1% (v/v) methanol which was filter sterilized and added to the cooled salts medium after autoclaving. Medium FDMY consisted of FDM medium in addition to 0.05% yeast extract (Difco). Medium FDV contained the basal salts plus 1% (v/v) Wolfe's Vitamin solution (13). Medium FDMV contained the methanol and vitamin additions.

Media GS and GSV contained the basal salts in addition to 0.1% (w/v) glucose sterilized separately and added after cooling of the media. GSV also contained 1% (v/v) vitamin solution described above. Media CAS and CASV contained the basal salts medium in addition to 0.1% casamino acids (Difco). CASV medium contained the additional vitamin supplement. Solid media were prepared from the above media with the addition of 1.5% agar. All media were adjusted to pH 7.0 with NaOH.

Inocula

Inocula for growth and nutrition studies consisted of 3X washed cells which had been grown on a rotary shaker at 30 C for 48 hours. The inoculum size was 0.1 ml of a cell suspension having an OD_{660} of approximately 0.15. Methanol-utilizing isolates were grown in FDM medium, and heterotrophic isolates were grown on PPYE medium.

Nutrition Studies

Each 18 mm tube which contained 10 ml of medium was inoculated and incubated at 24 C for nutritional studies, or at 30 C on a rotary shaker for

methanol experiments, or at the indicated temperatures for the temperature experiments. Tubes were rubber stoppered, when the medium contained volatile materials. Carbon sources were added at 0.5 or 0.1% (w/v) except for gaseous alkanes which were present in the tube atmosphere at 20% (v/v). For methanol utilizing organisms, nitrogen sources were added at levels of 0.1% (w/v) to the FDM medium from which nitrate was deleted. To test various carbon sources, the FDM medium minus methanol was used as a basal medium.

General conditions of cultivation

Stock cultures of the ZCA isolate were maintained on FDV agar plates incubated in a desiccator which contained an 80:20 air : methane atmosphere.

Cultures of the methanol-utilizing organism were maintained on FDM slants whereas the heterotrophic isolates were maintained on PPYE slants.

Determination of poly-β-hydroxybutyrate

The method of Law and Slepecky (6) was used.

Enumeration of floc members

A sample of water of homogenized floc (1.0 ml) was serially diluted in FD medium. A floc sample was prepared by homogenization of a weighed portion (0.2 g) of floc in 10 ml FD medium with 20 strokes in a hand pyrex homogenizer. Duplicate plates of PPYE, FDM and FDV media were prepared by spreading with a glass rod 0.1 ml of an appropriate sample dilution on each plate. All plates were incubated at 22 C in an air atmosphere except for FDV plates which were incubated in an 80:20 air : methane atmosphere. Colonies which developed on the media were counted at 5 and 10 days.

Mixed-culture, cross-feeding studies (liquid media)

Each cross-feeding experiment was carried out in a 25 ml Delong flask (Morton closure) which contained 5 ml of FDV medium. For inoculation

of ZCA organism 0.2 ml of a 7-day-old FDV liquid culture was used. When MOH-2 organism was used, an inoculum of 0.1 ml was prepared from an appropriate dilution of washed cell suspension of 48-hour-old culture from FDM medium. Flasks were incubated at 30 C in a glass dessicator which contained an air:methane ratio of 80:20 atmosphere. MOH-2 cell numbers were determined by duplicate plate count on FDM medium at 0 and 7 days.

Cross-feeding studies on agar plates

Methane isolate (ZCA) interactions with heterotrophic isolates (III--2, 3, 4, 5, 6, 7) were carried out on FDV medium. Organisms were inoculated on streak plates as shown in figures 9a, b, c. The inoculum of ZCA isolate was from a four-day-old FDV plate culture, each heterotrophic inoculum was prepared from a culture which had been grown on PPYE medium for 24 hours, the cells being washed twice in FD medium. Viability of heterotroph inoculum was demonstrated on PPYE medium whereas inability of these organizms to utilize FDV medium was demonstrated on FDV medium (figure 9c). Incubation of plates was at 22 C. Plates were examined periodically for development of growth.

Methanol isolate (MOH-1 and MOH-2) interactions with heterotrophic isolates (III--2, 3, 4, 5, 6, 7) were carried out on FDM medium. All inocula consisted of washed cells resuspended in FD medium: heterotrophic isolates were grown 24 hours in PPYE medium while methanol-utilizing organisms were grown 24 hours in FDM medium. Plate-streak procedures were identical to the methane cross-feeding experiments described above.

RESULTS

Figure 1 is a photograph of the flocculent deposits in their natural state in the Sullivan flush tank. Such material develops at the air-water interface producing copious amounts of slime. The flocs are pink to rust in color. Floatation is due to gas bubbles under the flocs or to attachment to the tank walls. When the tank is flushed, the flocs ride down and remain on the surface as the fresh water fills the tank. The flocs may reach dimensions of 8 to 10 square cm and 0.3 cm in thickness before breaking into smaller units. With increasing size the flocs often become unstable to floatation; they sink into the tank and are washed out of the system.

Examination of the floc material by phase contrast microscopy (fig. 2) reveals the material to be of microbial origin. Several morphological cell-types can be seen in the dense cell population. Examination at higher magnification (fig. 3) shows the predominant morphological cell type to be a large ellipsoidal rod as indicated by the arrow. A second cell type, slender rods, can be seen in the upper left corner of the photomicrograph. Refractile inorganic deposits are often seen but do not appear to constitute a major proportion of the floc.

A series of electron-scan micrographs prepared from floc taken directly from the tank can be seen in figures 4a, b, c. As seen in the low power micrograph (fig. 4a) the exterior surface of the floc is continuous. In b and c numerous cells can be seen embedded in the extracellular matrix.

Electron micrographs of sectioned floc material is presented in figures 5 a, b. The predominant organism (fig. 5a) contains elaborate extensive internal membrane systems. This ellipsoidal rod possesses a typical Gram-negative cell wall and also appears to contain inclusion vacuoles as evidenced by the electron transparent areas. Lysed cells on the left of the photograph have pulled away from the extracellular matrix. The other morphologically distinct cell type seen in floc electron micrographs is shown

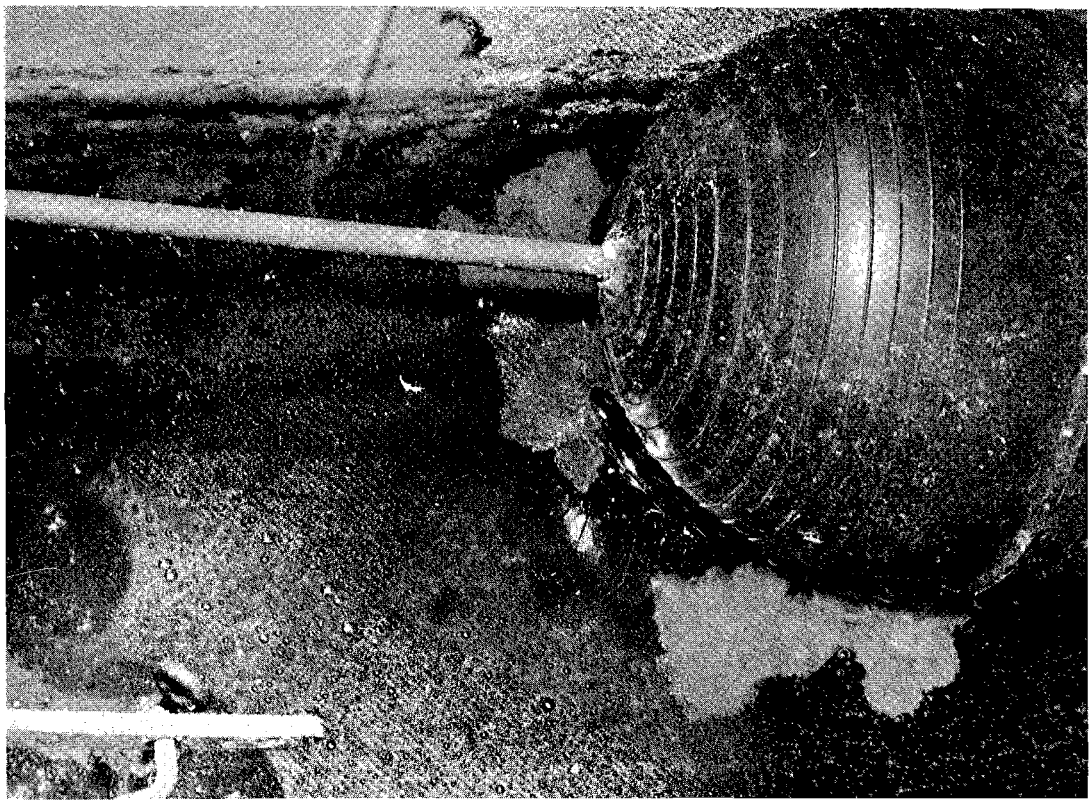


Figure 1. Photograph of the Sullivan flush tank showing development of flocs at the water surface.

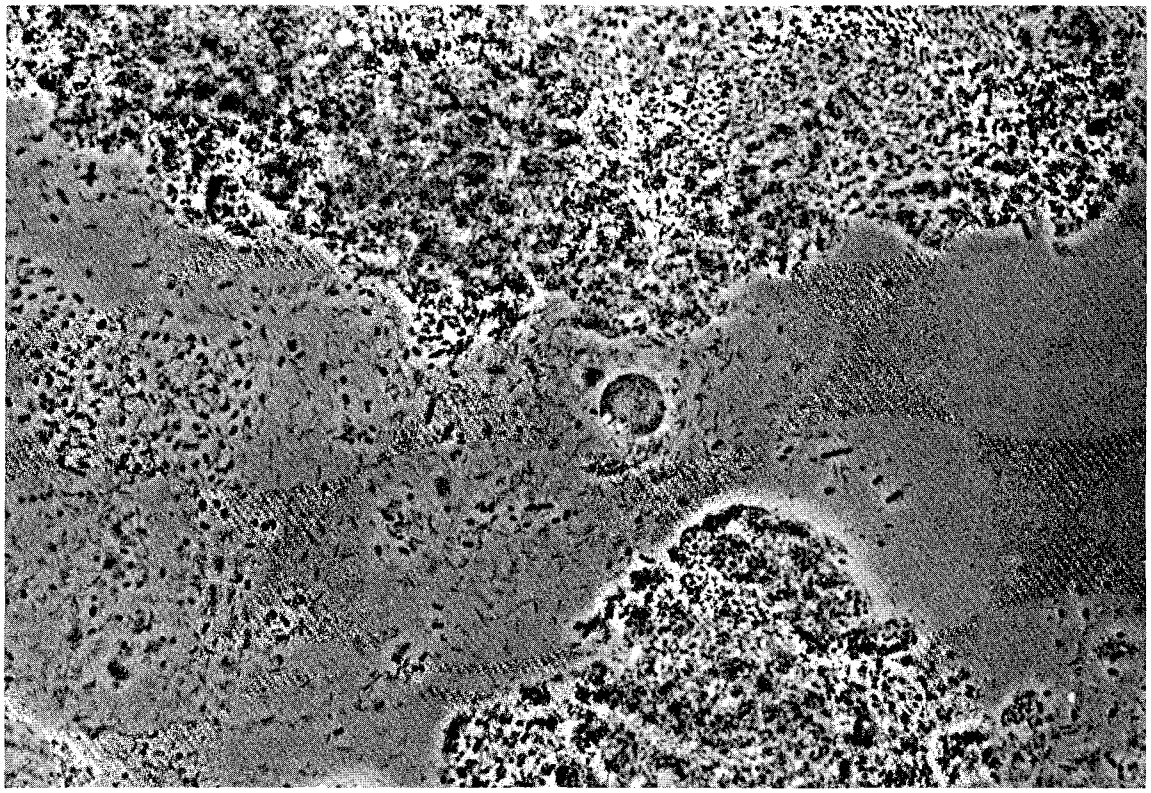


Figure 2. Phase contrast photomicrograph of the floc consortium showing the heterogenous microbial population. (X400)

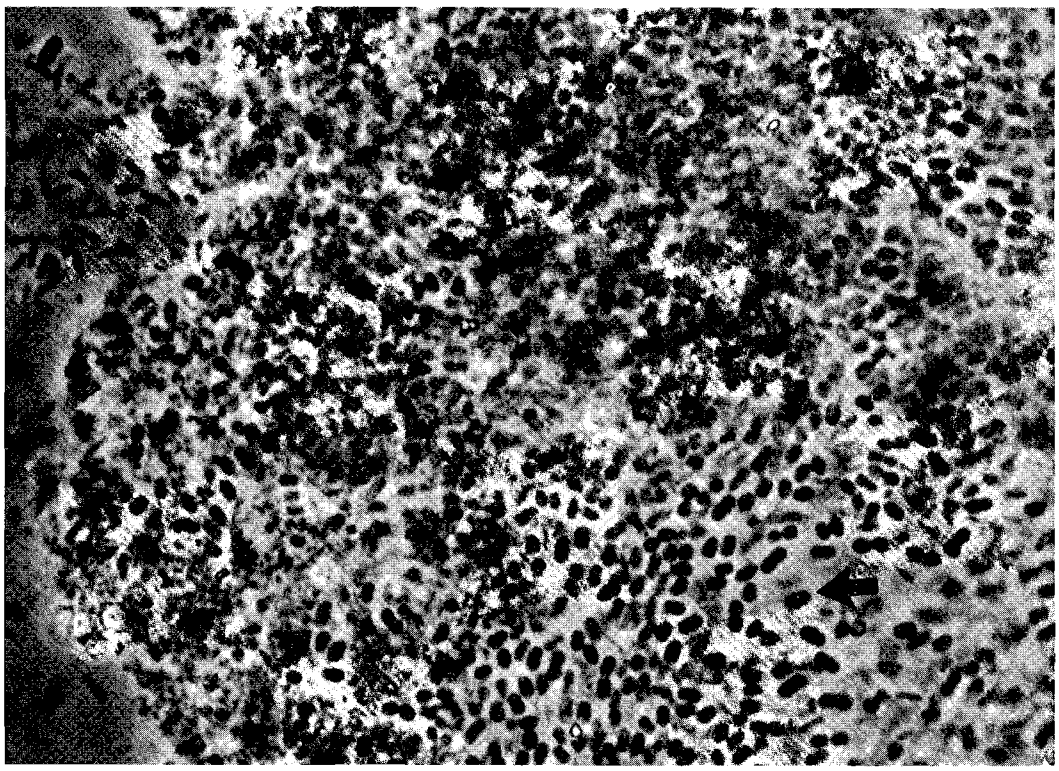
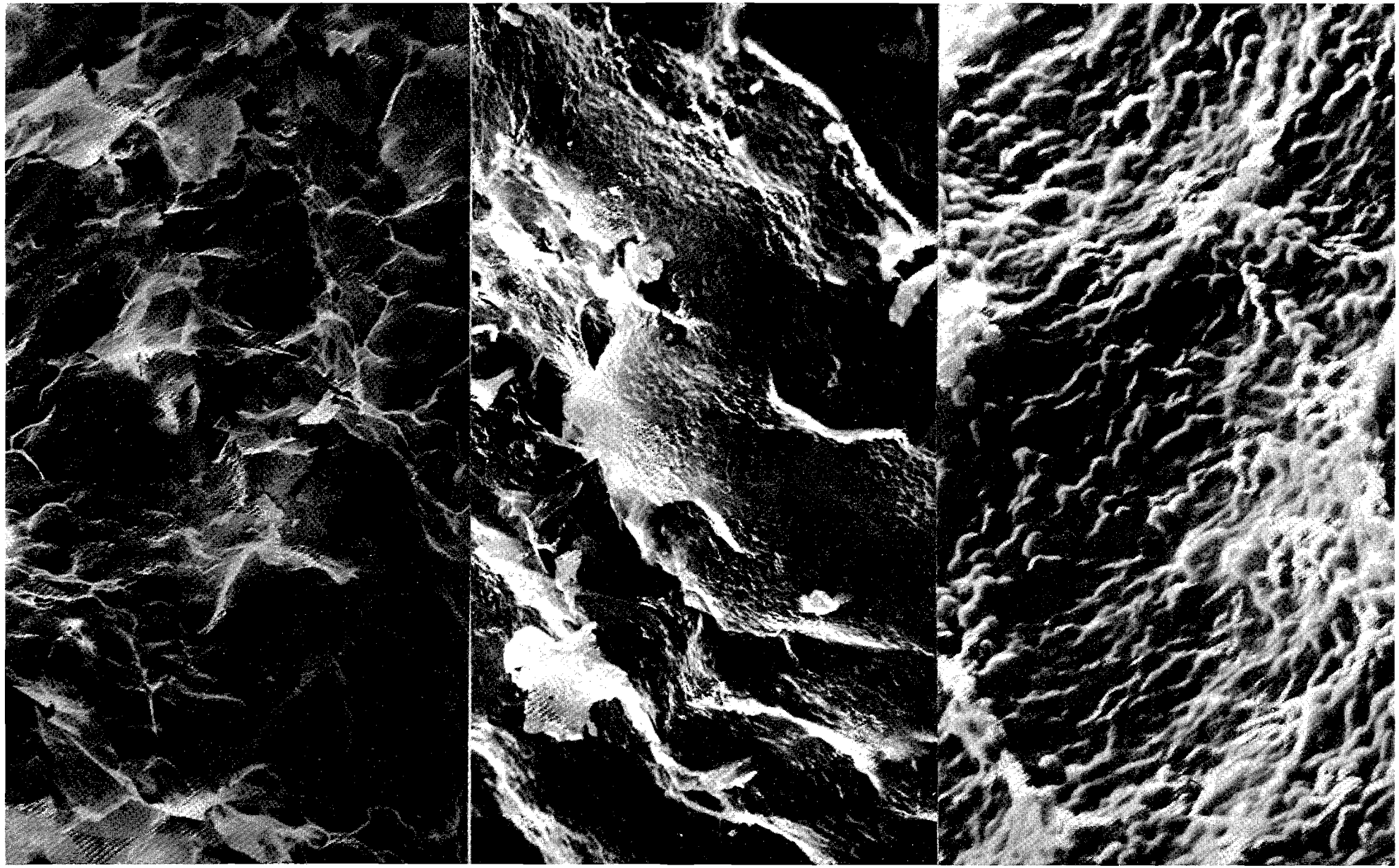


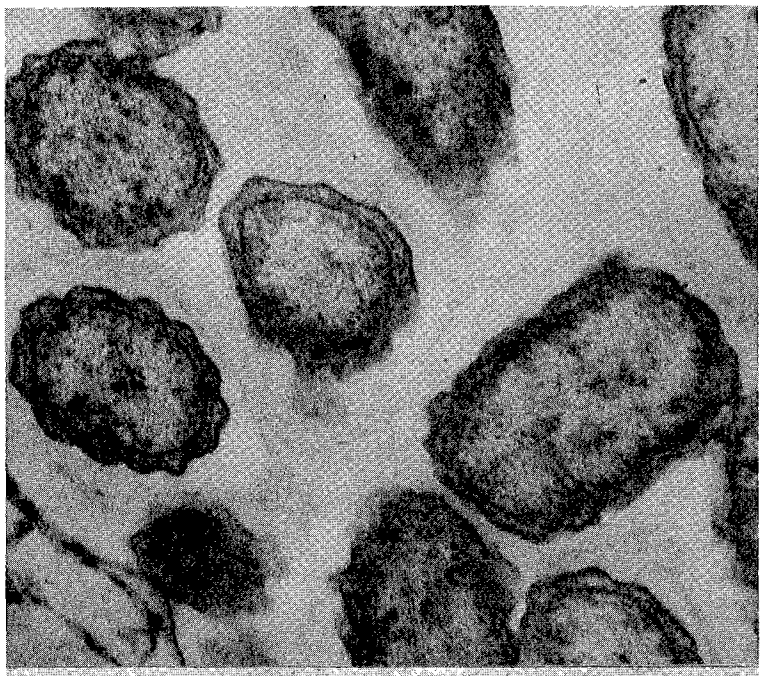
Figure 3. Phase photomicrograph of the consortium illustrating the principal morophological cell type. (X1200)



a. b. c.

Figure 4a, b, c. A series of scanning electron micrographs prepared from floc taken from the tank. (X250, X1500, X5,500)

a.



b.

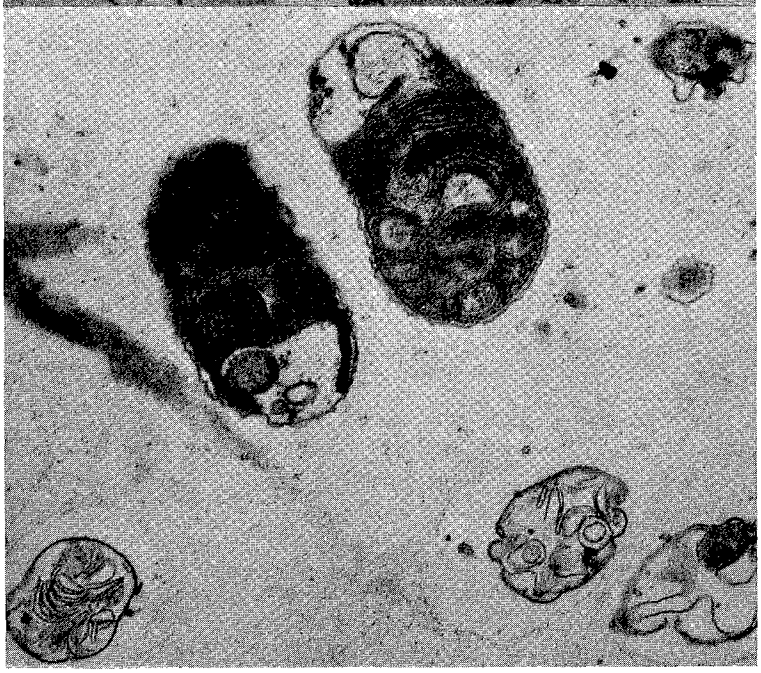


Figure 5a. Electron micrograph of floc; thin section showing typical predominant organism composing the consortium. (X20,100)

Figure 5b. Electron micrograph of floc; thin section showing the second predominant morphological cell type observed in the consortium. (X36,900)

in fig. 5b. These organisms lack the internal membrane system but do possess an interesting Gram-negative cell wall. A capsular matrix surrounds both morphological cell types. Such material accounts for the flocculent nature of the bacterial deposits. Examination of low-power electron micrographs of the floc interior reveals areas of lysed cells as well as debris still embedded in the matrix. The individual cell types multiply to form micro colonies and do not appear to contain cells of differing morphology. This cellular matrix is an apparent barrier to microbial migration.

To better understand the nutritional basis of these microbial consortia chemical analysis of the water was undertaken. Table 1 shows a representative chemical analysis of the well water supplied to the flush tanks. Total solids dissolved in the water represent 733 mg per liter of which 192 mg was volatile upon ashing. Organic material is evidently present only in low amounts. The water is slightly alkaline at pH 7.6 to 7.8 and has a temperature in the range of 20 to 23 C. Water in the flush tank is supplied from a private well drilled to bedrock at a depth of 154 feet. The pump is submerged in the well and feeds water to a 60 gallon reservoir which is maintained under positive pressure.

It was observed that an unusually large amount of gas was being released from the pressurized water system. Free gas bubbles can be seen in the flush tank (fig. 1). Collection and gas analysis was performed by the Illinois State Geological Survey. Table 2 shows the chromatographic analysis of a representative sample. Methane constitutes 67% of the total gas sample. Higher alkanes were absent. Oxygen and carbon dioxide were present at low levels of 0.6 and 0.7% respectively. It became evident that methane is the nutritional basis for the microbial consortium.

To understand the role of the organisms in the development of the consortium enrichment and isolation of these organisms was pursued.

Table 1. Represenative analysis of Sullivan well water.[a]

Determination	mg/l	Determination	mg/l
Iron	1.7	Phosphate	
Manganese	0.05	(filt.)	1.6
Aluminum	0.02	(unfilt.)	2.5
Calcium	28.0	Silica	14.7
Magnesium	12.2	Fluoride	0.2
Sodium	253.	Boron	0.3
Potassium	1.5	Nitrate	2.0
Ammonium	5.2	Chloride	138.
Copper	0.00	Sulfate	0.0
Nickel	0.05	Alkalinity	
Volatile Solids	192.	(as $CaCO_3$)	480.
Total Solids	733.		

[a]Analysis provided by the Illinois State Water Survey Laboratory.

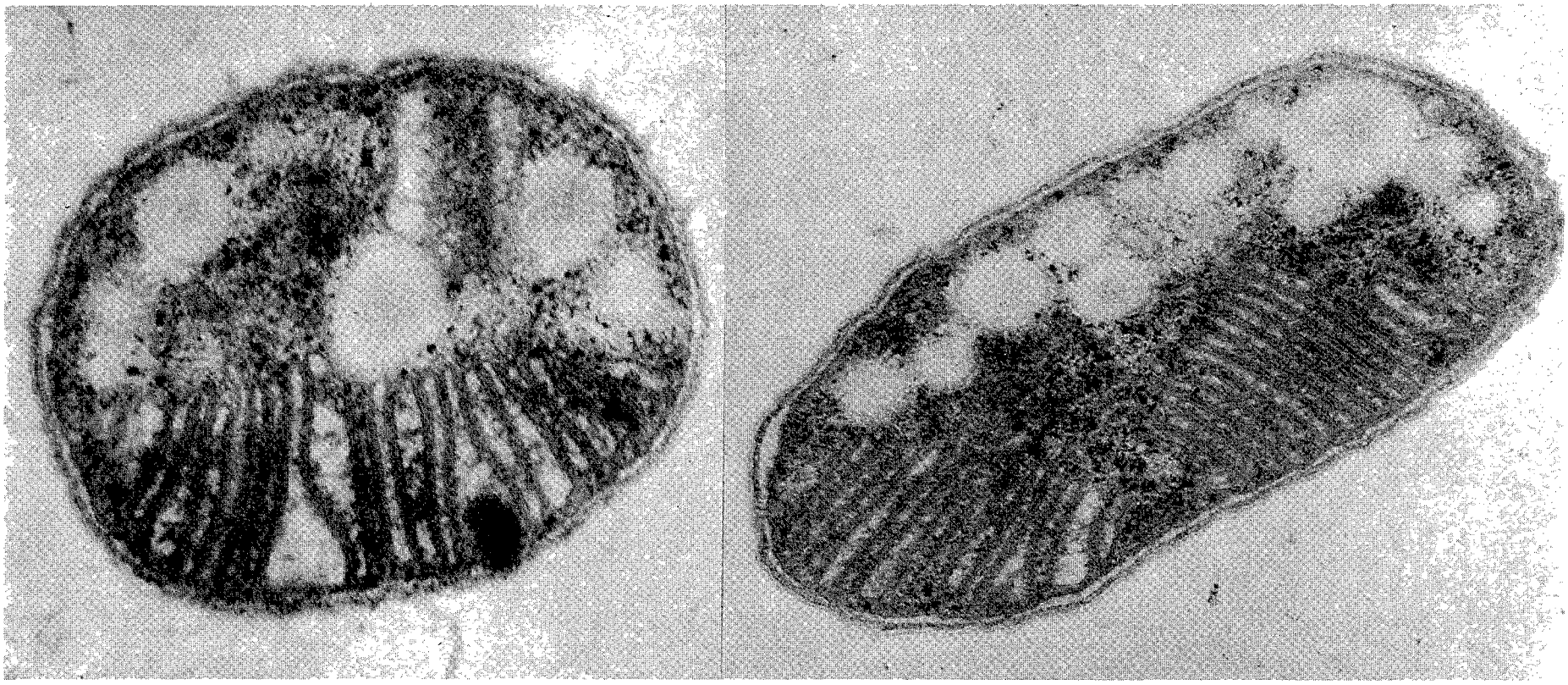
Table 2. Represenative analysis of Sullivan well gas.[a]

Determination	% v/v	Determination	% v/v
CO_2	0.6	Alkanes	
O_2	0.7	CH_4	66.9
CO	0.0	C_2H_6	0.0
H_2	0.0	C_3H_8	0.0
N_2	31.8	C_4H_{10}	0.0
He	0.0	C_5H_{12}	0.0
Ar	0.0	C_6H_{14}	0.0
		Total	100.0

[a]Analysis provided by the Illinois State G ological Survey.

Preliminary attempts to isolate the predominant organism, the large ellipsoidal rod, by use of various heterotrophic media were unsuccessful. Since gas analysis directed our attention to methane as the substrate which provided energy and carbon to the consortium, enrichments for methane-oxidizing bacteria were made in 500 ml Erlenmeyer flasks, each flask containing 50 ml of WS or WSY medium. Each flask was inoculated with 0.2 ml of either whole or homogenized floc prepared by disrupting about 1 cm^2 of the flocculent material with several strokes in a Pyrex glass homogenizer. Enrichments were gassed with an atmosphere of 80:20 percent (v/v) air:methane. The rubber-stoppered flasks were incubated at 24 or at 30 C. Turbidity developed in the WSY flasks in several days, but observation by phase microscopy did not reveal the morphologically predominant organism to be present. Hetertrophic organisms utilizing the yeast extract apparently predominated in these enrichments. However, WS enrichments produced growth in one to two weeks and observation revealed the large ellipsoidal rods to predominate. Attempts to isolate those organisms on WS agar plates which were incubated in an air methane atmosphere resulted in cultures which could not be subcultured more than 2 to 3 times. Addition of a vitamin solution or small amounts of yeast extract allowed successful cultivation and isolation of this methane utilizing isolate. This organism was designated ZCA and appears to be identical to the predominant organism seen in the microbial flocs.

A thin section of this organism examined by electron microscopy is shown in figures 6a and b. The isolate contains the internal membrane system characteristic of the floc organisms seen in fig. 5a. Storage vacuoles in the cytoplasm can be seen. Attempts to identify this material as poly-β-hydroxybutyrate have not been successful. The cells are Gram-negative and are poorly motile. They measure 1 by 1.3 microns in size. Exospores and cysts have not been observed. This isolate produces an extensive capsule and



a. b.

Figure 6a, b. Electron micrographs of double fixed thin section of the methane oxidizing isolate. (X83,300)

forms a pink pellicle in liquid culture. The cells develop well on a basal salts medium (supplemented with vitamins), when a methane air atmosphere is present. Vitamin B_{12} may be substituted for the vitamin mixture. Methanol is the only other substrate able to support growth as sole carbon and energy source. Higher alkanes and alcohols will not support growth. Proteose peptone-yeast extract media will not support growth and will inhibit development at levels of 0.5% (w/v). The isolate will reduce nitrate to nitrite and ammonia but will not grow anaerobically with nitrate as an alternative electron acceptor. It is oxidase positive, catalase positive and has a growth temperature maximum of 30 C.

Initial methane enrichments also contained a second morphological cell type. Attempts to isolate this organism on methane-supplemented media were unsuccessful. However, use of methanol in place of methane proved successful. Enrichments and isolations were performed by methods used for methane-oxidizing bacteria except that FDM and FDMY media were used. Two distinct methanol-oxidizing organisms were obtained by isolation on FDM streak plates. These two isolates appeared to be predominant members of the methanol floc enrichments and were designated MOH-1 and MOH-2. Morphologically the two isolates appear similar. They are Gram-negative rods 0.4 by 1.4 microns in size. MOH-2 is shown in thin section photomicrographs in fig. 7a. It lacks the internal membrane system of the methane organisms and is polarly flagellated as shown in negative stain in fig. 7b. MOH-1 does not produce pigment whereas MOH-2 produces a pale-yellow pigment which is insoluble in water; both organisms form round glistening colonies on FDM agar plates. Extensive capsule is produced by MOH-1 as demonstrated in slide preparations when the organism is mixed with India ink. Clumping of cells occurs in unshaken liquid culture, but can be dispersed by vigorous shaking.

a.

b.

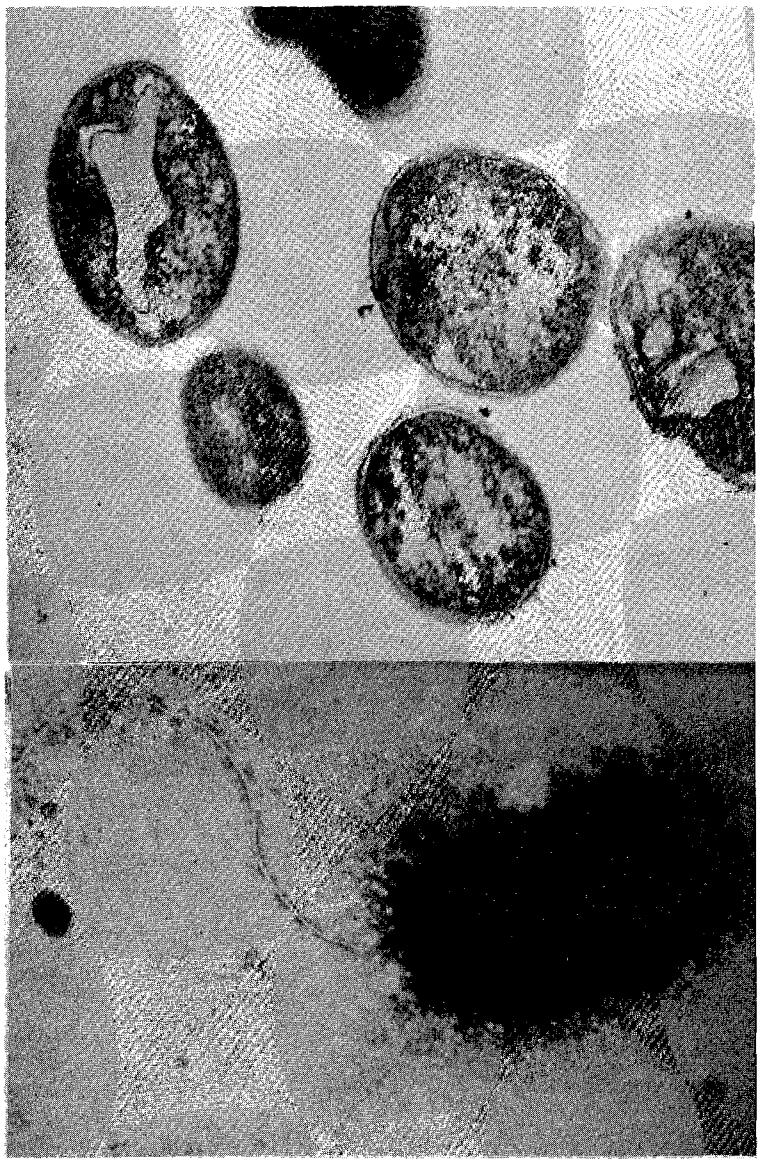


Figure 7a. Electron micrograph of thin section of methanol oxidizing isolate MOH-2. (X36,900)

7b. Negatively stained preparation of MOH-2 demonstrating polar flagellum. (X36,900)

Both organisms were catalase positive, are able to reduce nitrate to nitrite, but are unable to grow anaerobically with nitrate as an alternative electron acceptor. Although the isolates would not grow on yeast extract plates in the absence of methanol, yeast extract was not inhibitory at levels of 4% as long as methanol was present. The organisms differ in their ability to grow at different temperatures. MOH-1 grew optimally at 30 C but could grow at 37 C. MOH-2 had an optimal temperature of 23 C and could grow at 30 C. The effect of methanol concentration on growth also differed. MOH-1 grew in 4% methanol but was inhibited by 6%, whereas MOH-2 grew well in 2% but was inhibited by 4% methanol.

The organisms are able to grow only on methanol as sole carbon and energy source. The following alkanes, alcohols, organic acids, sugars and amino acids failed to support growth of either MOH-1 or MOH-2: methane, ethane, propane, butane, hexane, ethanol, propanol, butanol, oxalate, succinate, citrate, formate, butyrate, propionate, acetate, lactate, xylose, manitol, ribose, glucose, maltose, mannose, lactose, galactose, inositol, sucrose, cystine, leucine, asparaginine, glycine, valine, alanine, methionine, aspartic acid, and cysteine. Ammonium and nitrate salts when used as sole nitrogen sources allowed abundant growth of these organisms whereas amino acids were generally poor nitrogen sources.

Fifteen heterotrophic organisms were isolated from the consortium. Of these fifteen types, six can be routinely reisolated and appear to account for the majority of colonies developing on floc-inoculated heterotrophic media. The properties of these aerobic isolates are shown in Table 3; all are Gram-negative, non-motile rods. They do not produce noticeable capsule, are unable to reduce nitrate to nitrite and are catalase and oxidase positive with one exception. The ability of these isolates to grow on a number of different media is shown in Table 4.

Table 3. General characteristics of the major heterotrophic isolates.[a]

Isolate Designation	Cell Type	Colony Pigmentation	Gram Reaction	Motility	Capsule Production	Nitrate Reduction	Catalase	Oxidase
III-1	long rods	bright yellow	-	-	-	-	+	+
III-2	rods	dark yellow	-	-	-	-	+	+
III-3	rods	cream	-	-	-	-	+	+
III-4	short rods	pink	-	-	-	-	+	+
III-5	rods	cream	-	-	-	-	+	+
III-6	rods	white	-	-	-	-	-	+
III-7	long rods	off white	-	-	-	+	+	+

[a] -, negative reaction ; +, positive reaction

Table 4. Ability of the major heterotrophic isolates to utilize various media for growth.[a]

Isolate Designation	Medium[b]						
	FDV	FDMV	GS	GSV	CAS	CASV	PPYE
III-1	-	-	-	-	-	-	++
III-2	-	-	-	-	++	++	++
III-3	-	-	-	-	++	++	++
III-4	-	-	-	-	++	-	++
III-5	-	-	-	-	++	++	++
III-6	-	-	+	++	++	++	++
III-7	-	-	-	++	-	-	++

[a]Growth indicated as follows: -, negative responce; +, utilization; ++, greater utilization.

[b]Media contained the following components: FDV, basal salts, vitamin supplement and grown under an air:methane atmosphere of 80:20 % (v/v); FDM, basal salts with methanol; GS, glucose and basal salts; GSV, glucose, basal salts and vitamin supplement; CAS, casamino acids and basal salts; CASV, casamino acids, basal salts with vitamin supplement; PPYE, proteose peptone-yeast extract.

As a group they are unable to grow on methane or methanol as sole carbon source but require a more complex hetertrophic growth medium such as PPYE.

It has not been possible to determine absolute numbers of the various isolates composing the consortia. Disruption of the floc is difficult as capsule produced by the methane and methanol utilizing organisms causes cells to adhere in clumps which contain two to twenty or more cells. Further disruption of these particles leads to breakage of single cells. Thus plate counts of disrupted floc yield values which are low. A colony which develops on a plate may be initiated by more than one cell.

In an attempt to determine the significance of the heterotrophic population present in the floc a semi-quantitative enumeration of organisms was undertaken. By use of selective media it is possible to distinguish between the heterotrophic, the methanol, and the methane utilizing organisms on PPYE, FDM, and FDV media respectively. An enumeration of viable cells in a disrupted floc or in tank water is shown in Table 5. Practically no aerobic organisms are present in the well water supplied to the tank. Only after the water enters the tank does the population develop. The consortia may contain at least 10^8 to 10^9 viable cells per gram of floc. Both methane and methanol utilizing organisms are present at 10^9 cells per gram: actual numbers may be higher as colonies which develop on assay plates may be derived from more than one cell. The 10^4 values for cell populations present in tank water are not inconsistent with the observation that these organisms grow in a film at the air-water interface. It is this film of organisms which develops into macroscopic flocs. The important finding of the semiquantitative enumeration study is that significant numbers of methanol-utilizing as well as heterotrophic organisms develop in the tank environment, but they fail to develop on the same Sullivan well water in pure culture.

Table 5. Enumeration of organisms on various selective media. Values indicate minimal numbers of viable cells expressed as colonies developing per ml of water or per gram wet weight floc.

Sample Examined	Colonies Developing on Selective Media[a]		
	PPYE	FDM	FDV[b]
Sullivan well water	10	6	0
Tank water sample A	3.6×10^4	1.2×10^6	2.0×10^4
Tank water sample B	5.0×10^4	1.4×10^5	1.9×10^4
Floc sample A	1.2×10^9	7.4×10^8	9.1×10^8
Floc sample B	6.6×10^8	1.0×10^9	1.3×10^9

[a] PPYE, FDM and FDV media are selective for heterotrophic, methanol utilizing and methane utilizing organisms respectively.

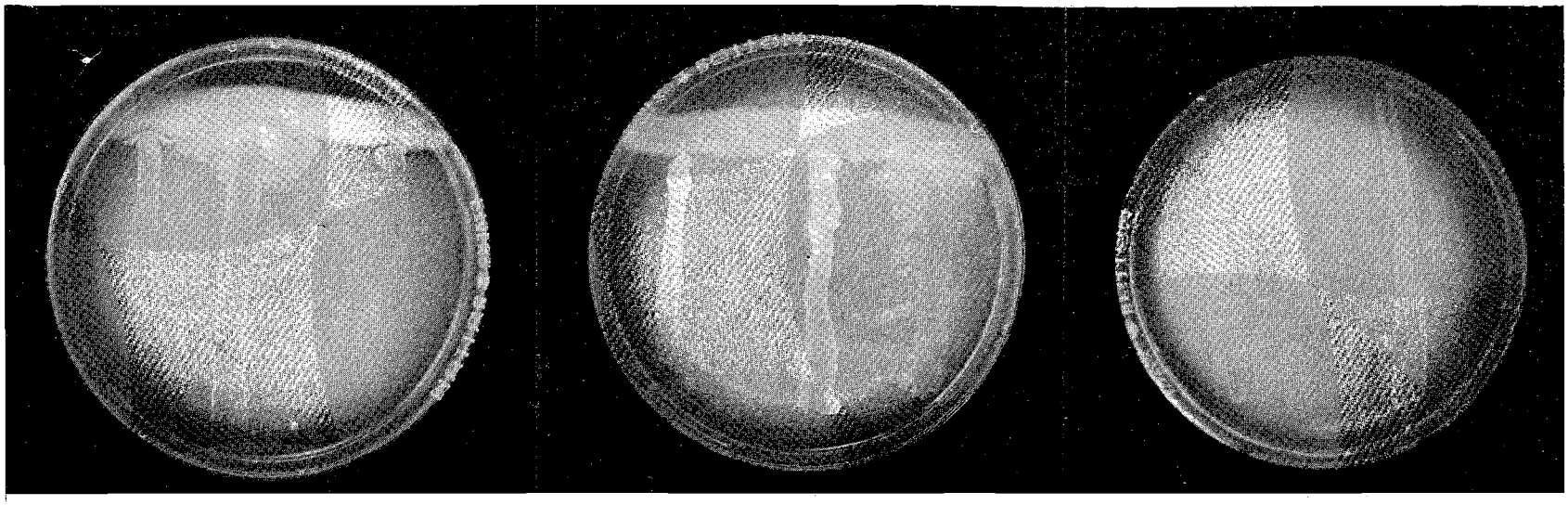
[b] FDV medium incubated under an air: methane atmosphere of 80:20 % (v/v).

It appears that a bacterial succession of organisms occurs in the consortium. Preliminary cross-feeding experiments with pure cultures of methane and methanol utilizing organisms indicate favorable pairings. Table 6 shows the increase in cell numbers of MOH-2 when grown in mixed culture with the methane oxidizing isolate (ZCA) on a medium which contained methane as the sole carbon and energy source. Flasks 1 and 2 contained inocula of MOH-2 and ZCA organisms. An increase of 10^1 and 10^3 MOH-2 cells per ml of medium occurred in seven days. Pure cultures of the non-methane-utilizing MOH-2 isolate when placed under identical conditions failed to produce growth as indicated by turbidity in three weeks. (Other attempts to grow this organism on methane have always failed.) Results of this experiment indicate that methanol or other decomposition products produced by the methane-utilizing isolate (ZCA) permit the growth of the MOH-2 organism when grown in pure culture. At present methanol is the only known substrate which will support growth of MOH-2.

Results of other cross-feeding experiments between the methane oxidizers and heterotrophic isolates as well as between the methanol-oxidizers and heterotrophic isolates also indicate favorable pairings of organisms. Figures 8a and b show examples of such cross-feeding experiments on streak plates. The heterotrophic organisms are streaked on the FDM medium which will not support their growth but will allow methanol utilizing isolates to develop. Materials produced by the methanol organisms diffuse into the agar and can be utilized by the heterotrophic organisms to support growth. After two weeks of incubation the cross-feeding is evident by development of the heterotrophic organisms as seen in the vertical streaks in figures 8a and b. Figure 8c shows an example of the very slight development of isolates III-2 and III-3 on FDM agar medium. Impurities in the agar are probably the source of nutrients, since no growth occurs in liquid medium.

Table 6. Increase in cell numbers of isolate MOH-2 when grown in mixed culture with the methane-oxidizing isolate (ZCA). FDV salts medium contained methane as sole carbon and energy source.

Flask Number	Number of viable MOH-2 cells per ml of mixed culture	
	At initial inoculation	After seven days
1	1.25×10^6	4.31×10^7
2	1.73×10^4	4.72×10^7



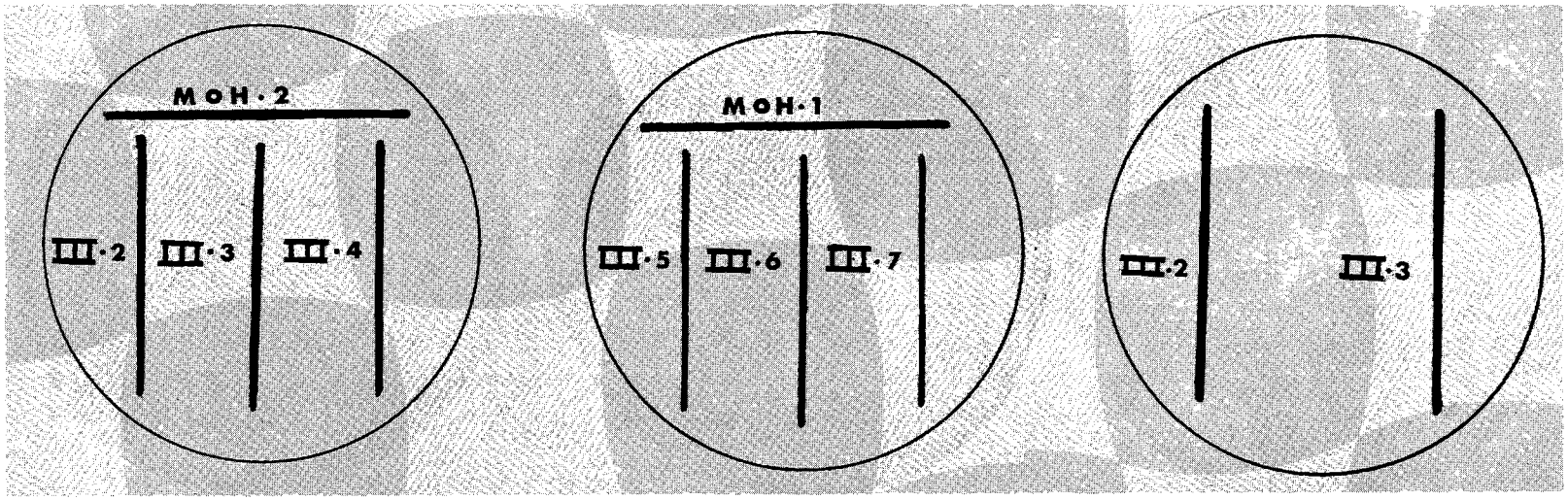


Figure 8. Photographs of typical cross feeding experiments on streak plates. The development of growth can be seen in figures 8a, b between the heterotrophs and methanol-utilizing organisms. Figure 8c shows the inability of two heterotrophs to develop on the same FDM medium at two weeks. The key below the photograph indicates the inoculation pattern on the plates.

Figure 9 shows the cross-feeding relationships between various isolates as determined by these streak plate tests. The lines joining the boxes indicate cross-feeding between the isolates designated in the boxes. It appears that both methanol-utilizing organisms (MOH-1 and 2) and the methane isolate (ZCA) will generally produce materials which can be utilized by the heterotrophs (III--2, 3, 4, 5, 6, 7). The dotted line between ZCA and MOH-1 indicates a pairing which has not been tried yet but may be similar to cross feeding between ZCA and MOH-2.

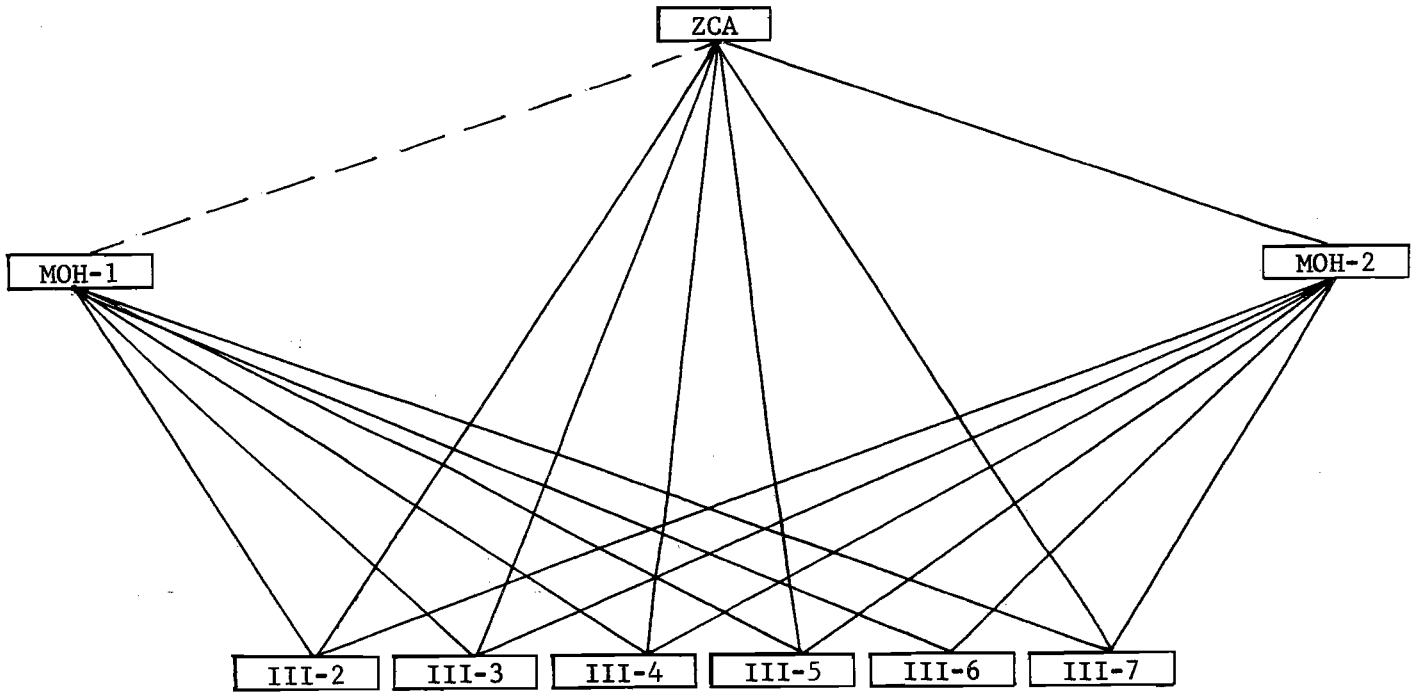


Figure 9. Diagram indicating pairs of consortium isolates between which cross feeding takes place as indicated by streak plates.

DISCUSSION

The flocculent material which develops in the Sullivan tanks has been shown to be of microbial origin. The oxidation of methane by the primary member of the consortium appears to provide the nutritional basis of the microbial consortium. A resulting community of organisms develops in these flocs leading to modification of water quality.

A number of organisms have been isolated from these flocs. A methane-oxidizing isolate has been obtained which produces an extensive capsule which is apparently responsible for the extensive slime deposited in the tank. This organism has taxonomic properties similar to those of Methylomonas methanica previously described by Whittenbury, et al., (12). A vitamin B_{12} requirement has been found for this strain. Two methanol-utilizing organisms can be routinely isolated from the consortia. One of these produces an extensive capsule which may contribute to the flocculent nature of the consortium. These organisms appear to have a unique nutritional requirement for methanol as sole carbon and energy source similar to that described by Dahl, et al., (3) for another obligate methanol utilizing organism. Their strain was able to oxidize several other compounds but only methanol supported growth. The cell wall ultrastructure of their organism differs from that of MOH-2; MOH-2 isolate may be a previously undescribed organism. A number of heterotrophic organisms also can be routinely isolated from the consortia and appear capable of growth on materials produced by the methane and methanol utilizing isolates from the consortium.

The solubility of methane as compared to other biologically important gasses as a function of temperature is shown in Table 7. Methane solubility may be decreased by several variables such as a decrease in pressure, increase in temperature and an increase of solute concentration (dissolved minerals) in the water. The result is a release of free gas. Both the drop in

Table 7. Solubility of methane compared to other biologically imortant gasses as a function of temperature. Data was calculated from Henry's constants (7). Values are expressed in mg of gas per liter at 760 mm pressure.

Temperature		Gas				
^{o}F	^{o}C	CH_4	O_2[a]	N_2	H_2	CO_2
32	0	39.7	70.4	29.5	1.92	3365
50	10	29.9	52.0	23.3	1.75	2348
68	20	23.7	45.0	19.4	1.63	1736
86	30	19.8	38.4	16.8	1.52	1290
104	40	17.1	33.8	14.9	1.48	1048
122	50	15.4	30.4	13.8	1.46	864

[a] O_2 solubilities under an air atmosphere are approximately 20% of these values as oxygen constitutes one fifth of the air atmosphere.

water pressure as the well water enters the flush tank and the slow increase in the cool water temperature to room temperature result in liberation of dissolved methane in the form of bubbles. The air-water interface in the tank is thus perfused with methane. At this oxygen rich surface, conditions are at an optimum for development of the consortium. Growth of this material is evidently limited by the diffusion of oxygen into the oxygen deficient tank water. A complex microbial community with high cell populations is able to develop in this nutrient rich well water.

The occurrence of methane in Illinois ground waters has been reported by Buswell and Larson (2). A state map showing areas containing methane in ground water has been reproduced and is shown in figure 10; the arrow indicates the location of the Sullivan site investigated in this report. Their 1937 study involving 35% of the state indicated methane present in 18 regions. There is no doubt that other areas are involved also. Studies currently under investigation by the Illinois State Geological Survey promise to document these and other areas in more detail.

In parts of the state methane is present at such levels that it has been tapped for commercial purposes. Meents (9, 10) reported locations of 460 glacial drift gas wells. These wells have yielded gas volumes of a few hundred to 1,700,000 cubic feet per day and have an average depth of 132 feet. Often the wells became flooded with water shortly after opening and could not be used. Drilling of water wells in many areas revealed associated gas. Methane content may range from free gas to barely detectable levels. Much of the glacial drift gas was reported to originate from buried soils, peats and organic rich silts associated with the interglacial stages which covered areas of the state 20,000 to 200,000 years ago. The occurrence of methane in Illinois ground waters appears to be a previously unrecognized source of energy for the development of microbial floc and subsequent modification of water qualities.

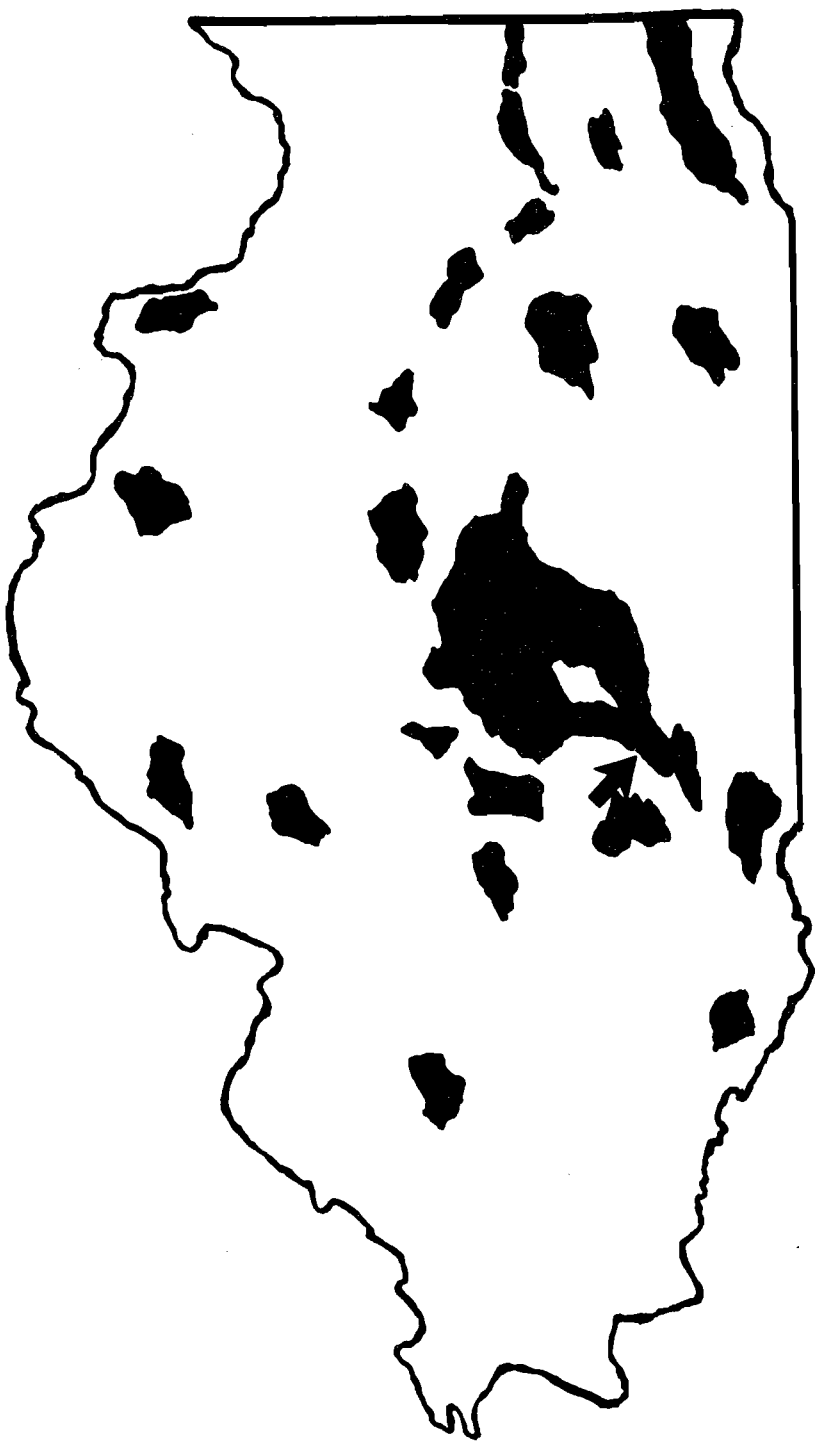


Figure 10. Illinois state map showing the distribution of methane in ground waters. Reproduced from Buswell and Larson (7).

Preliminary examination of a number of such flocs obtained from different areas of the state reveal them to be similar to the "model" consortium reported here. Isolation of both methane and methanol-utilizing organisms has been successful for six flocs so examined. These isolates appear morphologically similar to the predominant organisms seen in those flocs. Methane oxidation appears to be the basis for these typically pink to rust colored consortia also.

ACKNOWLEDGMENTS

We thank Mr. Laurel H. Henley and Mr. W. F. Meents for providing sample analysis in addition to helpful discussion concerning this project. We also wish to acknowledge Mr. M. Cantrell who worked on early portions of this project.

REFERENCES

1. American Public Health Association. 1971. Standard Methods for the Examination of Water and Wastewater. 13th ed., American Public Health Association, Inc., New York

2. Buswell, A. M., and T. E. Larson. 1937. Methane in Ground Waters. Journal of the American Water Works Association, 29: 1978-1982.

3. Dahl, J. S., R. J. Mehta, and D. S. Hoare. 1972. New Obligate Methylotroph. J. Bacteriol. 109: 916-921.

4. Foster, J. W., and R. H. Davis. 1966. A Methane-Dependent Coccus, with notes on Classification and Nomenclature of Obligate, Methane-Utilizing Bacteria. J. Bacteriol. 91: 1924-1931.

5. Kellenberger, E., A. Ryter, and J. Sechaud. 1958. Electron Microscope Study of DNA Containing Plasms. II. Vegetative and Mature Phage DNA as Compared with Normal Bacterial Nucleoids in Physiological States. J. Biophys. Biochem. Cytol. 4: 671-676.

6. Law, J. H., and R. A. Slepecky. 1961. Assay of poly-β-Hydroxybutric Acid. J. Bacteriol. 82: 33-36.

7. Loomis, A. G. 1928. Solubilities of Gases in Water. p. 255-261. In E. W. Washburn (ed.) International Critical Tables, Vol. 3, Mcgraw-Hill Co., Inc., New York.

8. Luft, J. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9: 409-414.

9. Meents, W. F. 1960. Glacial Drift Gas in Illinois. Circ. 292. Illinois State Geological Survey. Urbana, Illinois.

10. Meents, W. J. 1958. Tiskilwa Drift-Gas Area. Circ. 253. Illinois State Geological Survey. Urbana, Illinois.

11. Reynolds, E. S. 1963. The Use of Lead Citrate at High pH as an Electron Opaque Stain in Electron Microscopy. J. Cell Biol. 17: 208-212.

12. Whittenbury, R., K. C. Phillips, and J. F. Wilkinson. 1970. Enrichment, Isolation and Some Properties of Methane-Utilizing Bacteria. J. Gen Microbiol. 61: 205-218.

13. Wolin, E. A., M. J. Wolin, and R. S. Wolfe. 1963. Formation of Methane by Bacterial Extracts. J. Biol. Chem. 283: 2882-2886.