EVALUATION OF THE ANAEROBIC MICROORGANISMS AND THEIR METABOLISM IN AN ANOXIC LAKE BASIN

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ABSTRACT

EVALUATION OF THE ANAEROBIC MICROORGANISMS AND THEIR METABOLISM IN AN ANOXIC LAKE BASIN

By

John Joseph Molongoski

Strict anaerobic culture techniques were employed to quantitatively and qualitatively evaluate the anaerobic heterotrophic bacteria present at the sediment-water interface of Wintergreen Lake, a hypereutrophic hardwater basin. Anaerobic viable counts remained constant from March through December, 1973, ranging from 2.4 to 5.7 x 10^6 organisms per gram dry weight of sediment. These isolatable bacteria undoubtedly represented a small percentage of the total microbial community of these sediments, which was shown by direct microscopic counting techniques to be 2.0 x 10^{11} organisms per gram dry weight of sediment.

Bacteria of the genus Clostridium dominated the sedimentary isolates obtained, accounting for 71.8% of the 960 organisms examined. A single species, Clostridium bifermentens, comprised 47.7% of the total. Additional bacterial groups and the percentage in which they were isolated included: Streptococcus sp. (10.8%), Vibrio sp. (9.5%), Gram-positive nonsporing rods (5.6%), and motile Gram-negative rods (1.9%).

Considered as a whole, the isolates examined were

characterized by a high percentage of obligate anaerobes (85.7%) as compared to the number of facultative bacteria obtained (13.9%), and by the strikingly limited hydrolytic capabilities of these facultative organisms. The obligate anaerobes exhibited strong proteolytic, but weak saccharolytic capabilities. Temperature growth studies demonstrated the ability of all the isolates to grow at 10°C.

Gas-liquid-radiochromatography was employed to determine the gaseous and soluble metabolic endproducts produced from ^{14}C-labeled glucose and an amino acid mixture by representative sedimentary clostridial isolates and by a natural sediment microbial community. At in situ incubation temperatures, the natural sediment microflora produced soluble fermentative endproducts characteristic of those elaborated by the clostridial isolates tested. These results were considered strong presumptive evidence that clostridia are active in the decomposition of organic matter in the sediments of Wintergreen Lake.

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John Joseph Molongoski

A THESIS

Submitted to
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to my parents

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INTRODUCTION

The importance of anaerobic heterotrophic metabolism in lacustrine systems is emphasized by its involvement in all the major cycles of matter in natural waters (Ruttner, 1963; Deevey, 1970). Typically, lake sediments are recognized as having negative oxidation-reduction potentials as a consequence of the low solubility and diffusion of oxygen in water and its utilization by aerobic and facultatively anaerobic microorganisms in the water column and sediments (Brock, 1966). In the sediments, molecular oxygen is exhausted as an electron acceptor and anaerobic metabolism commences with the reduction of materials present in a highly oxidized state such as nitrate, ferric iron, and sulfate (Wetzel et al., 1972). Among the consequences of this process are increased solubility and diffusion of nutrients, including phosphate, ferrous iron, and silicate, from the sediments to the water often resulting in modified lake productivity (Mortimer, 1941 and 1942).

The majority of interest has centered on the reduction of inorganic nitrogen-, sulfur-, and carbon-containing compounds. The most important and interesting components of the sediments from a biological standpoint, however, are the organic substances, the microbial degradation of which provides the electrons for the above reductive processes to occur (Ruttner, 1963). The organic materials present in lake sediments undergo anaerobic decomposition in two or more distinct stages. In an initial non-methanogenic stage,

a heterogeneous group of facultative and obligately anaerobic bacteria convert proteins, carbohydrates, and lipids into an array of soluble and gaseous metabolic endproducts, including volatile short-chain fatty acids, alcohols, amines, ammonia, hydrogen sulfide, carbon dioxide, and hydrogen (Toerien and Hattingh, 1969). In a subsequent methanogenic phase, it was believed that the short-chain fatty acids and hydrogen resulting from the first stage were converted to methane and carbon dioxide by a physiologically distinct group of strictly anaerobic microorganisms termed the methanogenic bacteria. The latter observation has recently been challenged by Nelson and Zeikus (1974) who have demonstrated the conversion of $^{14}CO_2$ to $^{14}CH_4$ in incubated lake sediments, suggesting an autotrophic rather than a heterotrophic mode of metabolism for these bacteria. A third, intermediate group of anaerobic bacteria links the non-methanogenic and methanogenic stages by metabolizing volatile fatty acids and additional soluble organic endproducts to CO_2 and H_2 (Pine, 1971).

Regardless of the preferred mode of metabolism of the methanogenic bacteria, the production of methane in anoxic zones of natural waters serves as evidence for the decomposition of organic matter in these regions (Kuznetsov, 1968; Howard et al., 1971), as the production of the latter gas is dependent upon the initial anaerobic degradation of complex organic substrates. The quantity of organic matter carried in natural waters is small

compared to that present in the sediments (Stumm and Morgan, 1970). While aerobic metabolism removes much of the soluble organic material from the water column, the majority of the insoluble particulate and more refractory material falls to the bottom (Brock, 1966). This complex organic matter is metabolized by anaerobic heterotrophic bacteria, or is lost to the permanent sediments. The fermentation endproducts of these complex substrates in turn can suffer several fates. As already discussed, short-chain fatty acids, CO_2, and H_2 are presumptive precursors of methane formation in lake sediments. Alternatively, in hardwater lakes, soluble fermentation endproducts may be removed from the system by sorption to precipitating calcium carbonate particles (Wetzel, 1972; Kerr et al., 1973).

Quantitatively, the most significant fate of these metabolic endproducts may be their diffusion into the water column (Kerr et al., 1973) where they are readily utilized by a diversity of metabolically distinct bacteria. Acetate and H_2S, for example, serve as electron donors for certain purple and green sulfur bacteria (Pfennig, 1967). Alternatively, these soluble reduced compounds can be oxidized by groups of aerobic bacteria which are present in oxygenated portions of the water column (Kerr et al., 1973). In laboratory microcosm studies, Otsuki and Hanya (1972a; 1972b) have demonstrated that up to four times as much dissolved organic matter is released from decaying

green algal cells under anaerobic conditions as compared to aerobic conditions. In nature, then, a considerable amount of dissolved organic matter is likely liberated from lake sediments as a consequence of the anaerobic decomposition of particulate and complex soluble organic materials.

Determinations of the bacterial communities of lake sediments have historically involved the enumeration of the total number of cells present by direct counting procedures, or the enumeration of viable cells by plating techniques, utilizing media previously determined to yield the greatest number of bacterial colonies under specified conditions (Rodina, 1972; Sorokin and Kadota, 1972). More recently, the distribution and density of specific physiological groups of microorganisms in sediments such as sulfate reducers, denitrifying bacteria, or anaerobic heterotrophs have been determined by employing media presumably designed to selectively enumerate each of these groups of bacteria (Menon et al., 1971; Vanderpost and Dutka, 1971; Dutka et al., 1974). However, few attempts have been made to isolate and characterize these organisms, to determine their sensitivities to molecular oxygen, and to further elucidate their role in sedimentary metabolism.

Collins (1962) and Mah and Sussman (1967) have pointed to the need for the analysis of the bacterial communities of freshwater habitats under oxygen-limiting conditions. Nevertheless, workers in aquatic microbiology

have been slow to apply the techniques of strict anaerobic bacteriology originally developed to study other strictly anaerobic environments (Hungate, 1950; Aranki et al.,1969) to investigations of the unique anaerobic habitats of natural waters.

Attempts have also been made to study the metabolic activities of heterotrophic bacteria in sediments by measuring the rate at which they convert uniformly labeled organic substrates to $^{14}CO_2$ (Kadota et al., 1966; Harrison et al., 1971; Hall et al., 1972; Okutani et al., 1972). Few of these studies were conducted under anaerobic conditions, in spite of the anoxic nature of most lake sediments. Boylen and Brock (1973) compared the incorporation of ^{14}C (U)-glucose into cell material and the conversion of the labeled substrate into $^{14}CO_2$ under aerobic and anaerobic conditions. They obtained similar values for the incorporation of labeled glucose into cell material under both conditions, but found that less $^{14}CO_2$ was produced under anaerobic as compared to aerobic conditions. However, as bacterial fermentation is by definition incomplete, it is necessary to employ techniques which measure the gaseous and soluble fermentative end-products produced in order to account fully for the fate of uniformly labeled organic substrates incubated anaerobically in the presence of natural sediment microbial communities.

The present study represents an attempt to quantify and characterize the predominant groups of strictly anaerobic heterotrophic bacteria present at the sediment-water interface of a eutrophic lake, utilizing the strict anaerobic techniques developed by Hungate (1950) and Aranki et al (1969). An effort was also made to elucidate the role of natural sediment microbial communities in the anaerobic degradation of organic materials and the production of dissolved organic matter by utilizing gas-liquid-radiochromatography to assess the gaseous and soluble metabolic endproducts resulting from the catabolism of uniformly labeled organic substrates by these communities.

MATERIALS AND METHODS

Study Site

All investigations were conducted on Wintergreen Lake, a small hardwater basin located within the W. K. Kellogg Bird Sanctuary, Kalamazoo County, Michigan, R. 9W., T. 1N. Sec. 8. The lake has an area of 14.98 ha., a maximum depth of 6.3 meters, and a mean depth of 3.54 meters (Manny, 1971). Annual mean primary productivity values identify Wintergreen Lake as hypereutrophic, the latter designation based on compression of the trophogenic zone to a point where light rather than available nutrients limit productivity (Wetzel, 1966; Manny, 1971). Wintergreen Lake is characterized by a hypolimnion which is anoxic for nearly 7 months of the year, with anaerobic conditions extending to 3 meters during midsummer stratification. All sediment samples secured during this study were taken at a depth of 6 meters or greater.

Temperature

The temperature of the sediment-water interface was measured at approximately two week intervals from March through December, 1973. The *in situ* temperature was determined with a Model 46 TUC tele-thermometer (Yellow Springs Instr. Co., Yellow Springs, Ohio), the probe of which was weighted to insure its penetration into the sediment.

Dissolved Oxygen

Water samples were collected for dissolved oxygen analysis on the same dates that sediment temperature was determined and bacteriological analyses were performed. Samples were taken at 1 meter intervals with a 3 liter Van Dorn water sampler and subsamples were siphoned into 300 ml BOD bottles. The samples were immediately fixed in the field and returned to the laboratory where the dissolved oxygen concentration was determined by the Winkler technique (Standard Methods for The Examination of Water and Wastewater, 1971). Duplicate titrations were performed on each subsample.

Bacteriological Sampling Procedures

Sediment samples for bacteriological analysis were collected with a Plexiglass gravity corer (8.5 cm by 110 cm) that preserved the sediment-water interface. Samples were collected at approximately monthly intervals from March through December, 1973. Cores were immediately plugged with air-tight rubber stoppers for transport to the laboratory where a subsample was taken using a sterile glass subsampler (3 cm by 23 cm). The subsample was continually gassed with a mixture of 90% N_2-10% H_2 or 85% N_2-5% CO_2-10% H_2 before being plugged with rubber stoppers, clamped into a press, and transferred into an anaerobic glove box (Aranki et al., 1969). During the initial period of the study, the anaerobic glove box contained an atmosphere of 90% N_2-10% H_2. The gas composition of the

chamber was later altered to 85% N_2-5% CO_2-10% H_2. No significant differences were noted in the types or numbers of bacteria isolated under the two gas atmospheres.

An initial 1:10 dilution (vol/vol) of the surface sediment (0-2 cm layer) was made in pre-reduced anaerobic dilution fluid. The composition of the dilution fluid varied with the gas phase employed (Table 1). The initial 1:10 dilution was either stirred on a magnetic stirrer for five minutes, or mixed in a Waring blender for 1 minute. Appropriate additional dilutions were made in anaerobic dilution fluid and pre-reduced agar plates containing 0.05% resazurin as an oxidation-reduction indicator were inoculated from several different dilutions of the sediment. Media inoculated included 1% peptone-yeast extract-glucose (PYG), 0.5% PYG, Brain Heart Infusion (BHI, Difco, Detroit, Michigan), sediment extract supplemented with trypticase, yeast extract, and mineral salts, and Medium 10 of Caldwell and Bryant (1966) modified for use under a predominantly N_2 atmosphere. 1% PYG was pre-determined to yield the highest counts of bacteria and was routinely used for all quantitative viable counts. The complete composition of each medium is listed in Table 2.

Inoculated plates were incubated anaerobically at 15°C for 14 days. In the initial portion of the study, a stainless steel anaerobic chamber maintained at 15°C was used to incubate the plates. After August, 1973, a vinyl glove box (Coy Manufacturing, Ann Arbor, Michigan)

Table 1. Composition of anaerobic dilution fluid utilized under different gas atmospheres

Atmosphere of 90% N_2-10% H_2

$(NH_4)_2SO_4$	0.05%
$MgSO_4 \cdot 7H_2O$	0.005%
NaCl	0.1%
K_2HPO_4	0.65%
KH_2PO_4	0.35%
Cysteine-HCl$\cdot H_2O$	0.05%
0.1% Resazurin soln (vol/vol)	0.2%
pH adjusted to 6.9	

Atmosphere of 85% N_2-5% CO_2-10% H_2

K_2HPO_4	0.65%
KH_2PO_4	0.35%
Na_2CO_3	0.25%
Cysteine-HCl$\cdot H_2O$	0.05%
0.1% Resazurin soln (vol/vol)	0.2%
pH adjusted to 6.9	

Table 2. Composition of the media utilized in the isolation of anaerobic bacteria from Wintergreen Lake sediments

% in medium (wt/vol)

Component	1% PYG	0.5% PYG	BHI	Sediment Extract	Modified Medium 10
Peptone	1.0	0.5			
Yeast Extract	1.0	0.5		0.4	
Glucose	1.0	0.5			0.5
Cellobiose					0.5
Soluble Starch					0.5
Trypticase				0.2	0.5
Hemin (vol/vol)					1.0
Volatile Fatty Acids (vol/vol)					0.31
Agar	1.5	1.5	1.5	1.5	1.5
Salts Soln 1 (vol/vol)	90	90	90		90
Salts Soln 2 (vol/vol)	10	10	10		10
Salts Soln 3 (vol/vol)				2.0	
Difco Brain Heart Infusion			3.7		
Cysteine-HCl·H_2O	0.05	0.05	0.05	0.05	0.05
0.1% Resazurin Soln (vol/vol)	0.4	0.4	0.4	0.4	0.4
Sediment Extract (vol/vol)				30*	

*Sterilized separately and added aseptically after autoclaving

The pH of each media except the sediment extract was adjusted to 7.8 before autoclaving; the final pH of the media after eqilibration under 85% N_2-5% CO_2-10% H_2 was 7.0; the pH of the sediment extract was adjusted to 7.0 before autoclaving and remained at 7.0 after equilibration under the anaerobic atmosphere.

Table 2 (continued)

Salts Solution 1 (per 900 ml)

K_2HPO_4	520 mg
KH_2PO_4	280 mg
Na_2CO_3	200 mg
$MgSO_4 \cdot 7H_2O$	100 mg
$(NH_4)_2SO_4$	200 mg
NaCl	480 mg
$FeCl_3$	5 mg

Salts Solution 2 (per 100 ml)

$CaCl_2 \cdot 2H_2O$	60 mg

Salts Solution 3 (per 1000 ml)

$(NH_4)_2SO_4$	1.0 g
$MgSO_4 \cdot 7H_2O$	0.1 g
NaCl	2.0 g
K_2HPO_4	13.0 g
KH_2PO_4	7.0 g

Volatile Fatty Acid Mix

Acetic acid	17 ml
Propionic acid	6 ml
Butyric acid	4 ml
Valeric acid	1 ml
Iso-valeric acid	1 ml
Iso-butyric acid	1 ml

was used for all bacteriological procedures. Inoculated plates were placed in anaerobic gas-pak jars (BBL, Cockeysville, Maryland) containing a palladium catalyst. The jars were sealed and removed from the glove box to a 15°C incubator. After 14 days, the jars were returned to the glove box, opened, and the plates were examined qualitatively and quantitatively.

Characterization of Bacteria

All plates were carefully examined for colony diversity. Fifty to 60 colonies were randomly picked from each medium and transferred to 1% PYG plates. Although colonies were selected at random, an effort was made to insure that representatives of all distinct colony types present on the plates were included in the organisms transferred. The PYG plates were incubated at room temperature within the glove box. Greater than 95% of the isolates grew within three days. The colonies were then streaked onto 1% PYG plates and isolated colonies obtained were transferred to anaerobic PYG slants and refrigerated at 4°C until needed for further characterization.

The isolates were characterized primarily by the methods described in the Virginia Polytechnic Institute (VPI) Anaerobe Laboratory Manual (Holdeman and Moore, 1972). All characterization tests were performed using pre-reduced anaerobically sterilized (PRAS) media, except for the determination of oxygen sensitivity when

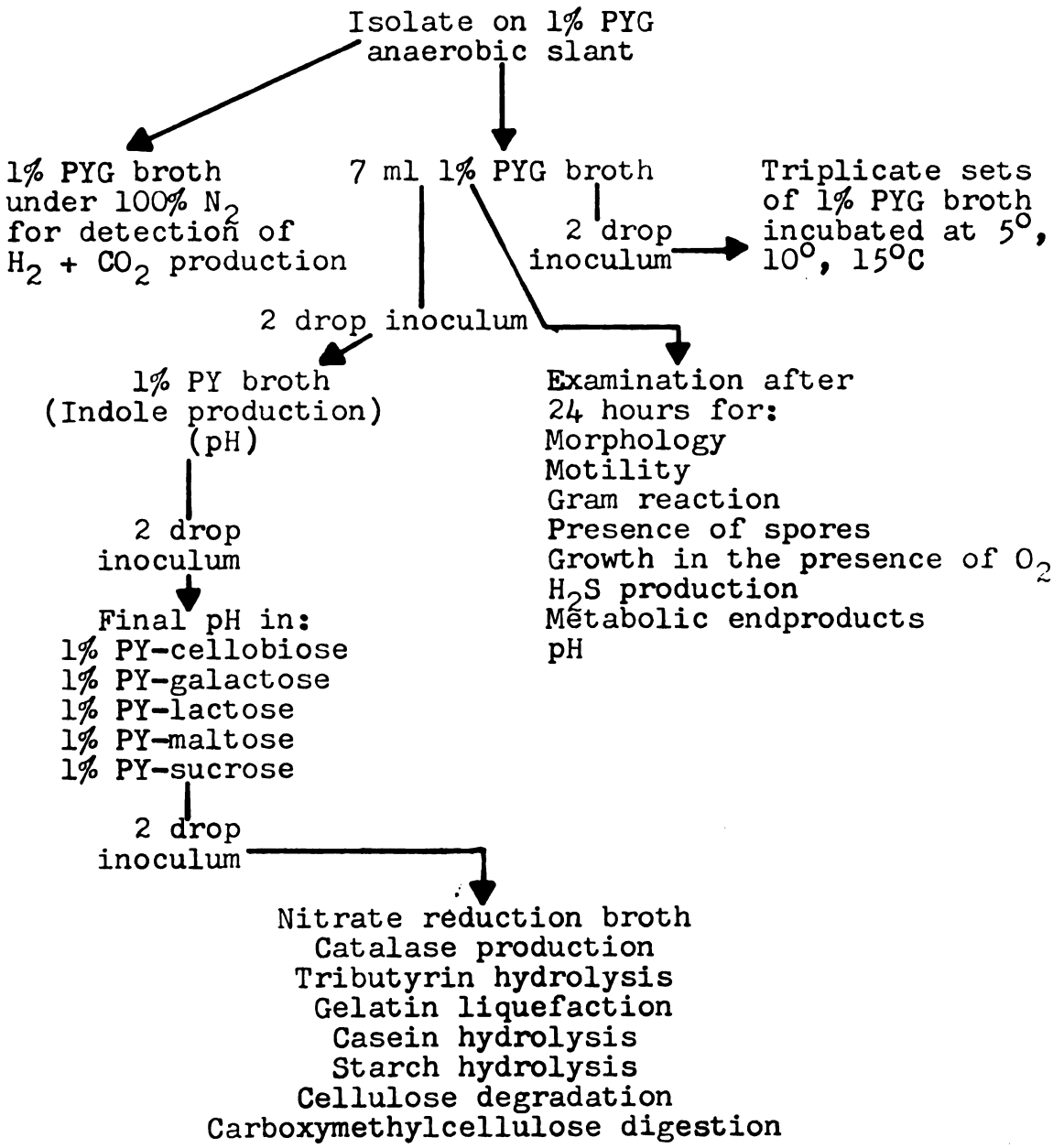
incubation was aerobic. In addition, the nitrate reduction broth described by Holdeman and Moore (1972) lacked cysteine in order to avoid chemical reduction of the nitrate in the medium. All characterization tests were performed at room temperature, except for the temperature growth studies.

In the characterization scheme utilized (Figure 1), 1% PYG was used as the basal medium with the various test substrates being added at a concentration of 1% (wt/vol). Morphology, motility, and the presence of spores were determined by examination of 18-24 hour PYG broth cultures by phase-contrast microscopy. In cultures in which no spores were apparent, spore production was determined by inoculating 1% PY-starch with 2-3 drops of the suspected culture, pasteurizing the stoppered tube for 10 minutes at 80°C in a water bath, and incubating the tubes at room temperature. Outgrowth within four days was scored as indicating positive spore formation (Holdeman and Moore, 1972).

Hydrogen sulfide production was determined by suspending a strip of Whatman # 1 filter paper previously soaked in 5% lead acetate within the headspace of each PYG broth. Black discoloration of the strip caused by the formation of lead sulfide indicated positive H_2S production.

In order to detect H_2 and CO_2 production by the individual isolates, tubes of 1% PYG broth were prepared under 100% N_2 utilizing the Hungate modification of the

Figure 1. Characterization scheme utilized in the identification of isolated sedimentary anaerobes



anaerobic roll tube as described by Macy et al., (1972). H_2 and CO_2 were analyzed by injecting 0.2 ml of the headspace of each culture into a Packard Model 409 gas chromatograph (Packard Instrument Co., Downers Grove, Ill.) equipped with a thermal conductivity detector. The column and operating conditions employed are listed in Table 3, part A.

The ability of each isolate to grow in the presence of molecular oxygen was determined by inoculating 1% PYG agar plates lacking cysteine with 1 drop of an actively growing broth culture of each organism, and incubating the plates aerobically at room temperature. Aerobic PYG plates were also streaked with each isolate. Both methods yielded identical results in nearly all cases.

The cellulolytic capabilities of the isolates were tested by incorporating 0.1% ball-milled cellulose (made from Whatman #1 filter paper and kindly supplied by Dr. M. P. Bryant) into pre-reduced PY plates. Zones of clearing around inoculated colonies indicated positive cellulolytic activity. Casein digestion and tributyrin hydrolysis were determined in a similar manner.

All additional tests, including indole production, nitrate reduction, Gram reaction, sugar fermentations, gelatin liquefaction, starch hydrolysis, catalase production, and endproduct analysis, were performed as outlined in the VPI Anaerobe Laboratory Manual (Holdeman and Moore, 1972). Volatile short-chain fatty acids

Table 3. Columns and conditions for the analysis of soluble and gaseous metabolic endproducts by gas-liquid and gas-solid chromatography

	Metabolites Analyzed	Column Utilized	Liquid Phase	Solid Support	Column Temp.	Detector Temp.	Injector Temp.	Carrier gas flow rate
A.	CO_2 + H_2	Stainless steel, 2 m x 2 mm ID		60/80 silica gel	50°C	60°C (TC at 150 ma)	130°C	N_2 at 25 ml/min
B.	Volatile short chain fatty acids	Glass, 2 m x 2 mm ID	10% SP-1200 -1% H_3PO_4	80/100 mesh AW-DMCS Chromosorb W	120°C	140°C (HFI)	120°C	He at 25 ml/min
C.	Volatile short chain fatty acids; Lactic acid	Stainless steel, 2 m x 2 mm ID	10% SP-1200 -1% H_3PO_4	80/100 mesh AW-DMCS Chromosorb W	120°C	150°C (HFI)	150°C	He at 25 ml/min
D.	CO_2, CH_4, and H_2	Stainless steel, 2 m x 2 mm ID		80/100 mesh Porapak	60°C	140°C (TC at 150 ma)	120°C	N_2 at 10 ml/min
E.	Volatile short chain fatty acids	Stainless steel, 2 m x 2 mm ID	7% FFAP	80/100 mesh AW-DMCS Chromosorb W	115°C	135°C (TC at 250 ma)	160°C	He at 20 ml/min

Symbols: TC, Thermal conductivity detector
HFI, Hydrogen flame ionization detector

Silica gel obtained from Applied Science Laboratories, State College, Pa.
SP-1200 obtained from Supelco, Inc., Bellefonte, Pa.
Porapak N obtained from Waters Associates, Framingham, Mass.
FFAP obtained from Varian-Aerograph, Walnut Creek, California

produced as metabolic endproducts by the isolates were analyzed on a Packard Model 409 or a Varian Model 600 (Varian Aerograph, Walnut Creek, Cal.) gas chromatograph equipped with hydrogen flame ionization detectors. Columns and operating conditions utilized are given in Table 3, parts B and C. When the fatty acids were analyzed on a stainless steel column, a glass liner was inserted into the injection port of the gas chromatograph in order to insure that the samples were volatilized on glass and not lost by adsorption to the column. Lactic acid was determined by converting it to its propyl ester (boron trifluoride-propanol esterification reagent obtained from Anspec Co., Ann Arbor, Michigan) and analyzing it by gas chromatography as described in Table 3, part C.

Metabolism of Labeled Organic Compounds by Selected Bacterial Isolates

Two representative isolates obtained from the sediments of Wintergreen Lake, and tentatively identified on the basis of fermentation endproducts and additional biochemical tests as Clostridium bifermentens and C. sporogenes, were selected for additional metabolic experiments. Each organism was grown in pre-reduced PYG broth. Two drops of a 24-hour culture of each isolate were added to 2 ml of 1% PYG broth containing 1 μCi/ml of ^{14}C (U)-D-glucose (Amersham/Searle, Arlington Heights, Ill.) and to 2 ml of 1% PY broth containing 1 μCi/ml of ^{14}C (U) -L- amino acid mixture (New England Nuclear,

Boston, Mass.) respectively. The composition of the labeled amino acid mixture is given in Table 4.

Immediately after inoculation, the culture tubes were sealed with butyl rubber septa and incubated for 24 hours at room temperature. The gaseous phase of each tube was analyzed for labeled CO_2 by injecting 0.2 ml of the culture headspace into the Packard Model 409 gas chromatograph equipped with a thermal conductivity detector. Column and operating conditions are given in Table 3, part D. A one eighth inch (OD) section of stainless steel tubing was swaged (Swagelok Fittings, Grand Rapids Valve and Fitting Co., Grand Rapids, Mi.) onto the thermal conductivity effluent port of the gas chromatograph such that the radioactive effluent passed directly into a Packard Model 325 Tri-carb combustion furnace (Packard Instru. Co.). The latter contained a quartz glass tube filled with copper oxide which was maintained at a temperature of 750°C. Organic components of the gas chromatographic effluent gas were thereby combusted to CO_2, passed through a magnesium perchlorate water trap, and the radioactive effluent collected at selected time intervals by bubbling it into a scintillation vial containing 9 ml of methanol, 6 ml of scintillator (15g PPO, 1 g bis-MSB, made up to 1 liter with toluene), and 4 ml of purified monoethanolamine (Packard Instrument Co.). The scintillation vials were counted in a Beckman Model LS-150 liquid scintillation spectrometer.

Table 4. Composition of ^{14}C (U) -L- amino acid mixture utilized in labeling experiments

^{14}C (U) -L- amino acid	millicuries per millimole	microcuries amino acid per millicurie mixture
Alanine	120	80
Arginine	256	70
Aspartic Acid	172	80
Glutamic Acid	215	125
Glycine	84	40
Histidine	282	15
Isoleucine	258	50
Leucine	258	140
Lysine	252	60
Phenylalanine	423	80
Proline	215	50
Serine	140	40
Threonine	188	50
Tyrosine	387	40
Valine	215	80

The clostridial cultures were centrifuged for 10 minutes in a clinical centrifuge. The supernatant from each was acidified with 3 drops of 50% H_2SO_4, extracted twice with 2 ml of redistilled ethyl ether, and concentrated under N_2 to a volume of 0.1 ml. A 10 µl sample of each extract was injected into the Packard Model 409 gas chromatograph. Operating conditions are given in Table 3, part E. Radioactive endproducts were chromatographed, combusted to CO_2, trapped in ethanolamine, and counted as described above for the gaseous phase, except that for the analysis of the soluble endproducts, the stainless steel tubing connecting the gas chromatograph to the combustion furnace was wrapped in asbestos heating tape (Cole-Parmer Co., Chicago, Ill.) and maintained at a temperature of 210°C to insure that sample condensation did not occur during passage from the gas chromatograph to the combustion furnace. An efficiency of approximately 50%, measured by extracting a sample of ^{14}C-acetate of known activity with ether and injecting it into the chromatograph, was determined for the system.

Metabolism of Labeled Organic Compounds By A Natural Sediment Microbial Community

The anaerobic metabolism of uniformly ^{14}C-labeled amino acid mixture and uniformly ^{14}C-labeled glucose by a natural sediment microbial community was also examined. Sediment samples were collected in the summer of 1974 using the same anaerobic collection techniques as

described previously. A 1:10 (vol/vol) dilution of sediment (0-2 cm layer) was made in the anaerobic glove box with pre-reduced filter-sterilized interstitial water (pH 7.2) which had previously been extracted from Winter-green Lake sediments by centrifugation at 15,000 rpm for 30 minutes. Ten ml of the 1:10 sediment dilution were added to duplicate 50-ml flasks containing 4 μCi/ml of pre-reduced ^{14}C (U) -D- glucose and 3.4 μCi/ml of pre-reduced ^{14}C (U) -L- amino acid mixture respectively. Cold glucose (0.115 mg/ml) and cold amino acid mixture (Sigma Chemical Co., St. Louis, Mo.) were also added to the respective flasks as carrier substrate. Control flasks were killed by the addition of 1 ml of 2% $HgCl_2$ or 1 ml of 6N H_2SO_4 before addition of sediment. The flasks were sealed with rubber serum bottle stoppers, removed from the glove box, and incubated with shaking for 72 hours at 10°C. The latter temperature closely approximated the actual temperature of the sediments at the time of sampling.

The reactions were terminated after 72 hours by injecting 1 ml of 6N H_2SO_4 into each flask. The flasks were allowed to shake an additional hour at 10°C to allow CO_2 release from the sediment to occur. After equilibration to room temperature, the gas phase of each flask was analyzed for radioactive components as described previously. The contents of each flask were extracted with successive 5-ml portions of ethyl ether. The ether

fraction was collected, concentrated to a volume of 0.1 ml under N_2, and a 10 ul sample was analyzed by gas-radio-liquid chromatography.

RESULTS

Quantitation of Anaerobic Bacteria at The Sediment- Water Interface

Figures 2 and 3 illustrate the density of anaerobic heterotrophic bacteria at the sediment-water interface and the concentration of dissolved oxygen in the water column of Wintergreen Lake from February through December, 1973. The density of anaerobic bacteria remained nearly constant throughout this period, with a moderate increase in June corresponding to the onset of intense anoxic conditions in the hypolimnion (Figure 3). The density of anaerobes at the sediment-water interface did not decline appreciably in November after lake turnover had occurred, a reflection of the near permanent anaerobiosis of these sediments and of the ability of the sediment microflora to withstand brief exposure to oxygenated conditions.

Temperature Of The Sediment-Water Interface

The temperature at the sediment-water interface of Wintergreen Lake from March through December, 1973 (Figure 4) varied considerably, ranging from a low of 3.4°C in December to a high of 13.3°C in October. Despite these seasonal flucuations, the measured sediment temperature remained 10°C or greater for a minimum of 6 months of the year.

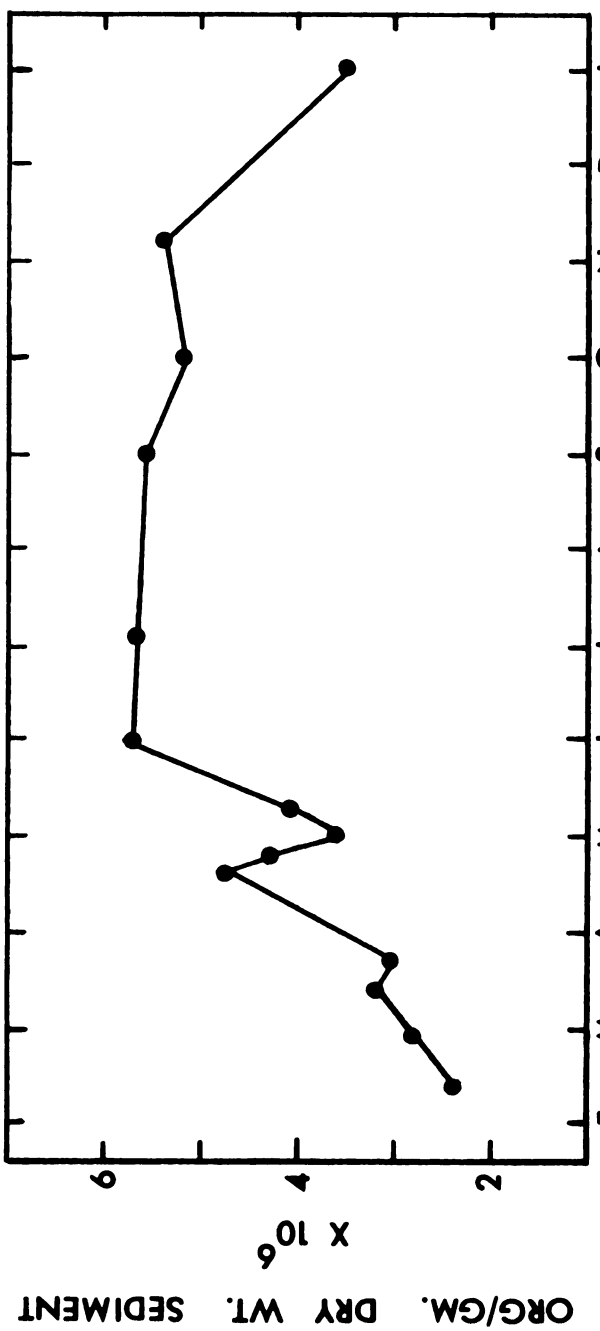


Figure 2. Anaerobic plate counts from the sediment-water interface of Wintergreen Lake, February through December, 1973.

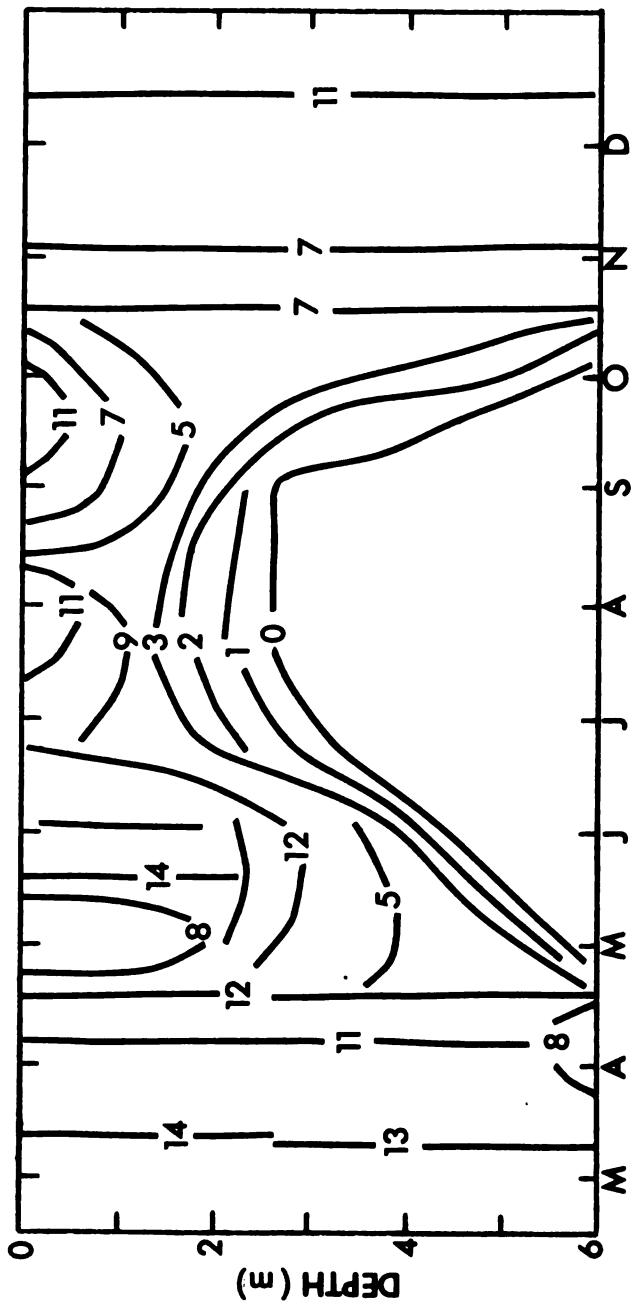


Figure 3. Depth-time diagram of the distribution of dissolved oxygen (mg/l) in Wintergreen Lake, March through December, 1973.

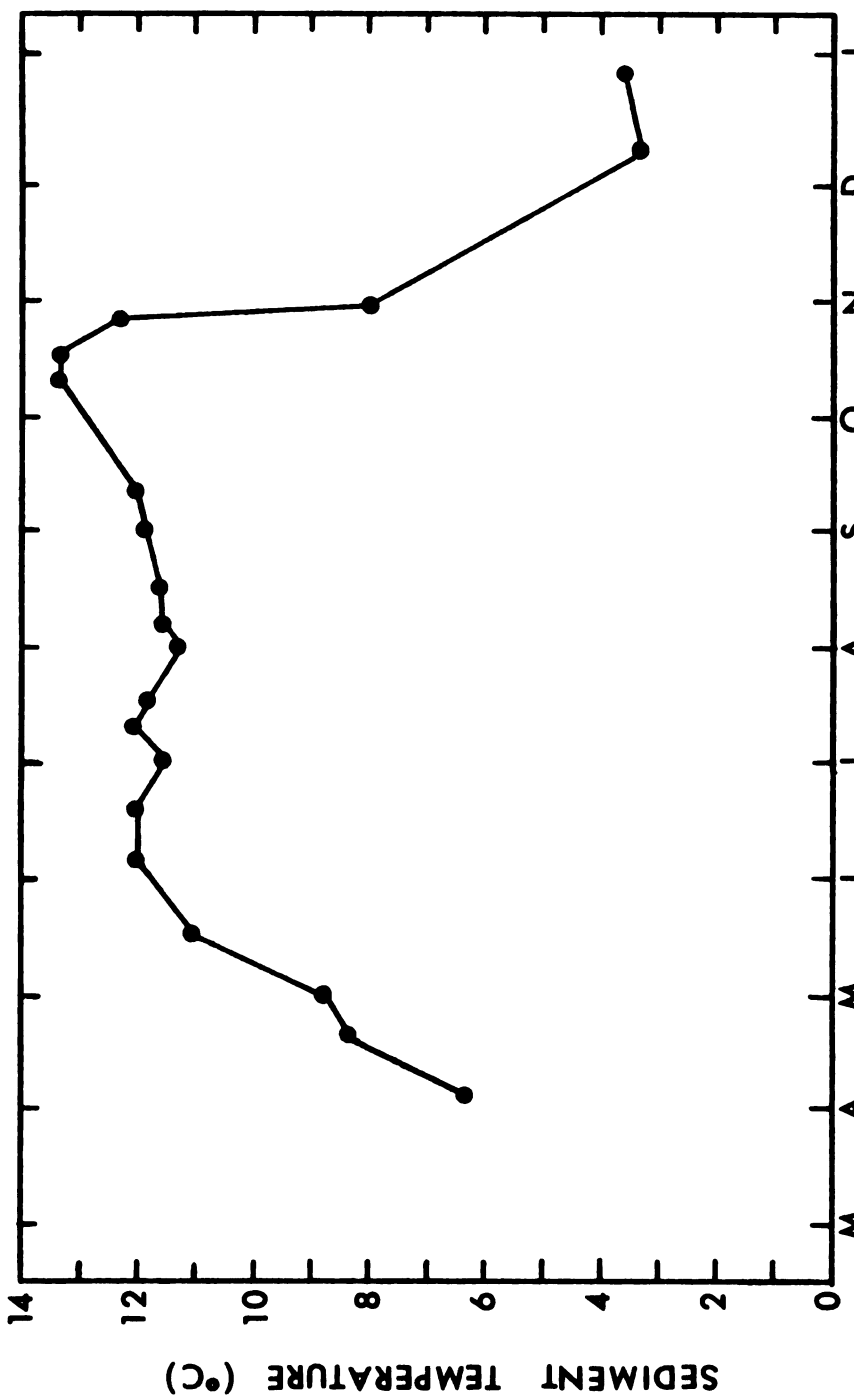


Figure 4. Sediment-water interface temperature at a depth of 6 meters in Wintergreen Lake, March through December, 1973.

Major Groups of Anaerobic Heterotrophic Bacteria Present At The Sediment-Water Interface

Table 5 summarizes the major groups of facultative and strictly anaerobic bacteria isolated from the sediment-water interface of Wintergreen Lake. Fermentation and additional physiological characteristics of each group are found in Table 6. Group I, tentatively identified as a Streptococcus sp., was composed of Gram-positive facultatively anaerobic cocci, 1-1.5 μm in diameter. The cells were either spherical or dumbbell shaped and occurred singly, in pairs, or in short chains. No H_2S or indole were produced, and fermentation endproducts in PYG included major amounts of lactic and acetic acids with trace quantities of propionic, butyric, and iso-butyric acids. Strains of Group I fermented all sugars tested, produced neither H_2 or CO_2 from PYG, and exhibited the ability to grow at 5°C. These isolates comprised 10.8% of the total organisms examined.

Group II, comprising 9.5% of the total isolates, consisted of motile Gram-negative curved rods measuring 0.5 by 3 μm. These organisms were obligately anaerobic, produced H_2S, and were indole-negative. Fermentation endproducts included major amounts of acetic and propionic acids with lesser quantities of butyric, iso-butyric, and iso-valeric acids. Presumptively classified as Vibrio species, these organisms fermented only maltose among the six sugars tested. These strains grew at 10° and 15°C,

Table 5. Presumptive identification features and distribution of bacterial groups isolated from Wintergreen Lake sediments

Group (Morphology)	Gram Reaction	Oxygen Tolerance	Fermentation products from PYG	Tentative identification	% of isolated strains*
I. Gram-positive cocci, 1-1.5 μm	+	F	LApbi-b	Streptococcus	10.8
II. Small curved rods, 0.5 x 3μm	-	A	APbi-b i-v	Vibrio	9.5
III. Spore-forming rods				Clostridium	71.8
a.	+	A	Apbi-b i-vi-c	C. bifermentens	47.7
b.	+	A	Apbi-b vi-v	C. sporogenes	17.8
c.	+	A	ApBi-B	C. butyricum	6.3
IV. Gram-positive rods					
a.	+	A	AB	Eubacterium	4.4
b.	+	F	ND	Undetermined	1.2
V. Motile Gram-negative rods, 0.5 x 2-3 μm	-	F	ND	Undetermined	1.9

*Based on a total of 960 isolates

Symbols: O_2 tolerance, A (Strictly anaerobic); F (Facultative) Fermentation products, A, a (Acetic); P, p (Propionic); B, b (Butyric); i-B, i-b (Isobutyric); V, v (Valeric); i-V, i-v (Isovaleric); i-C, i-c (Isocaproic); L, (Lactic) Upper case letters refer to major endproducts; lower case letters refer to products produced in lesser amounts. ND = not determined

Table 6. Fermentation and additional physiological characteristics of isolated sediment strains

Characteristic	Group Number*				
	1	2	3a	3b	3c
Glucose	a	n	w	w	a
Cellobiose	a	n	n	n	a
Galactose	a	n	n	n	a
Lactose	a	n	n	n	a
Maltose	a	a	w	w	a
Sucrose	a	n	n	n	a
Indole Production	-	-	+	-	-
H_2S Production	-	+	+	+	+
Catalase	-	-	-	-	-
Nitrate Reduction	-	-	-	-	-
H_2 Production	-	ND	+	+	+
CO_2 Production	-	ND	+	+	+
Growth at:					
5C	+	-	-	+	-
10C	+	+	+	+	+
15C	+	+	+	+	+

* Fermentation and additional physiological tests were performed on the first three groups of isolates only.

Symbols: a, acid reaction, terminal pH 5.5 or less
w, weak reaction, terminal pH 5.5-6.0
n, no fermentation, terminal pH greater than 6.0
ND, not determined

but not at 5°C.

By far the largest group of isolates obtained, representing 71.8% of the total, Group III consisted of anaerobic sporeforming rods of the genus Clostridium. This group could be further subdivided into three subgroups identified on the basis of fermentation endproducts and additional biochemical reactions as C. bifermentens, C. sporogenes, and C. butyricum. C. bifermentens represented the most predominant species obtained, comprising 47.7% of the total isolates.

The predominantly proteolytic nature of these clostridial isolates (Barker, 1961) was substantiated by their inability to ferment a variety of sugars, the sole exception being C. butyricum which was able to ferment all of the sugars tested. Also of note was the ability of the clostridial isolates to produce H_2 in PYG broth and their capability to grow at environmental temperatures. All three clostridial isolates grew at 10°C; C. sporogenes was able to grow at 5°C as well (Table 6).

Groups IV and V represented a minor portion of the isolates, together comprising only 7.5% of the total. Group IV consisted of Gram-positive nonsporing rods and included two subgroups, the first an obligately anaerobic rod which produced acetic and butyric acids as major fermentation endproducts and which may be related to a species of Eubacterium, and the second, as unidentified

facultatively anaerobic rod. Group V consisted of unidentified motile Gram-negative rods which comprised less than 2% of the total isolates. (Table 5).

Table 7 illustrates the hydrolytic capabilities of each group of isolates towards a number of complex substrates. No cellulolytic activity was demonstrable as shown by the inability of all of the groups to hydrolyze cellulose or carboxymethyl-cellulose. Moreover, only the presumptive Eubacterium, the group V isolates, and one subgroup of clostridia, C. butyricum, were able to ferment starch. Similarly, only two groups, the group V isolates, and the presumptive Eubacterium exhibited lipase activity as demonstrated by the ability to hydrolyze tributyrin.

The majority of the isolates, on the other hand, exhibited proteolytic capabilities. The proteolytic nature of the clostridia was evident from the ability of C. bifermentens and C. sporogenes to hydrolyze both casein and gelatin. Only C. butyricum failed to attack these compounds (Table 7). The remaining strictly anaerobic isolates, the group II Vibrio and the group IV Eubacterium, also demonstrated proteolytic capabilities, while among the facultative isolates, only the motile Gram-negative rods of group V were able to liquify gelatin (Table 7).

Table 7. Hydrolytic characteristics of isolated Wintergreen Lake sediment strains

Group	Casein Disgestion	Gelatin Liquefaction	Starch Hydrolysis	Carboxymethylcellulose Digestion	Cellulose Digestion	Tributyrin Hydrolysis
I. Facultative cocci	-	-	-	-	-	-
II. Anaerobic Vibrio	±	+	-	-	-	-
III. Clostridium						
a.	+	+	-	-	-	-
b.	+	+	-	-	-	-
c.	-	-	+	-	-	-
IV. Gram-positive rods						
a. Anaerobic	-	+	+	-	-	+
b. Facultative	-	-	-	-	-	-
V. Gram-negative Facultative rods	-	+	+	-	-	+

Metabolism Of Labeled Organic Compounds By Selected Bacterial Isolates

Figures 5 through 9 illustrate the results of the analysis of the gaseous and soluble metabolic endproducts produced by C. bifermentens and C. sporogenes when grown in the presence of ^{14}C labeled amino acid mixture or glucose respectively. Neither organism produced great amounts of $^{14}CO_2$ from the amino acid mixture (Figure 5), or from glucose (results not shown), even though both isolates had been previously demonstrated to produce CO_2 in PYG broth (Table 6). Both isolates incorporated label into an array of short-chain fatty acids when grown in the presence of the ^{14}C-amino acid mixture (Figures 6 and 7). The labeled compounds represented by the histograms in the latter two figures, and in Figures 8 and 9 as well, were separated chromatographically, using a thermal conductivity detector and the chromatographic conditions listed in Table 3, part E. The fatty acid peaks in Figures 6 through 9 are authentic endproducts produced by the two isolates as determined by the more sensitive hydrogen flame ionization detector (Table 3, part C). By comparison, both C. bifermentens and C. sporogenes incorporated far lesser amounts of label into the soluble fatty acid fraction when grown in the presence of ^{14}C-glucose (Figures 8 and 9). These observations appear to substantiate the proteolytic nature of these organisms, but as the specific activities of the individual fatty acid peaks were not determined,

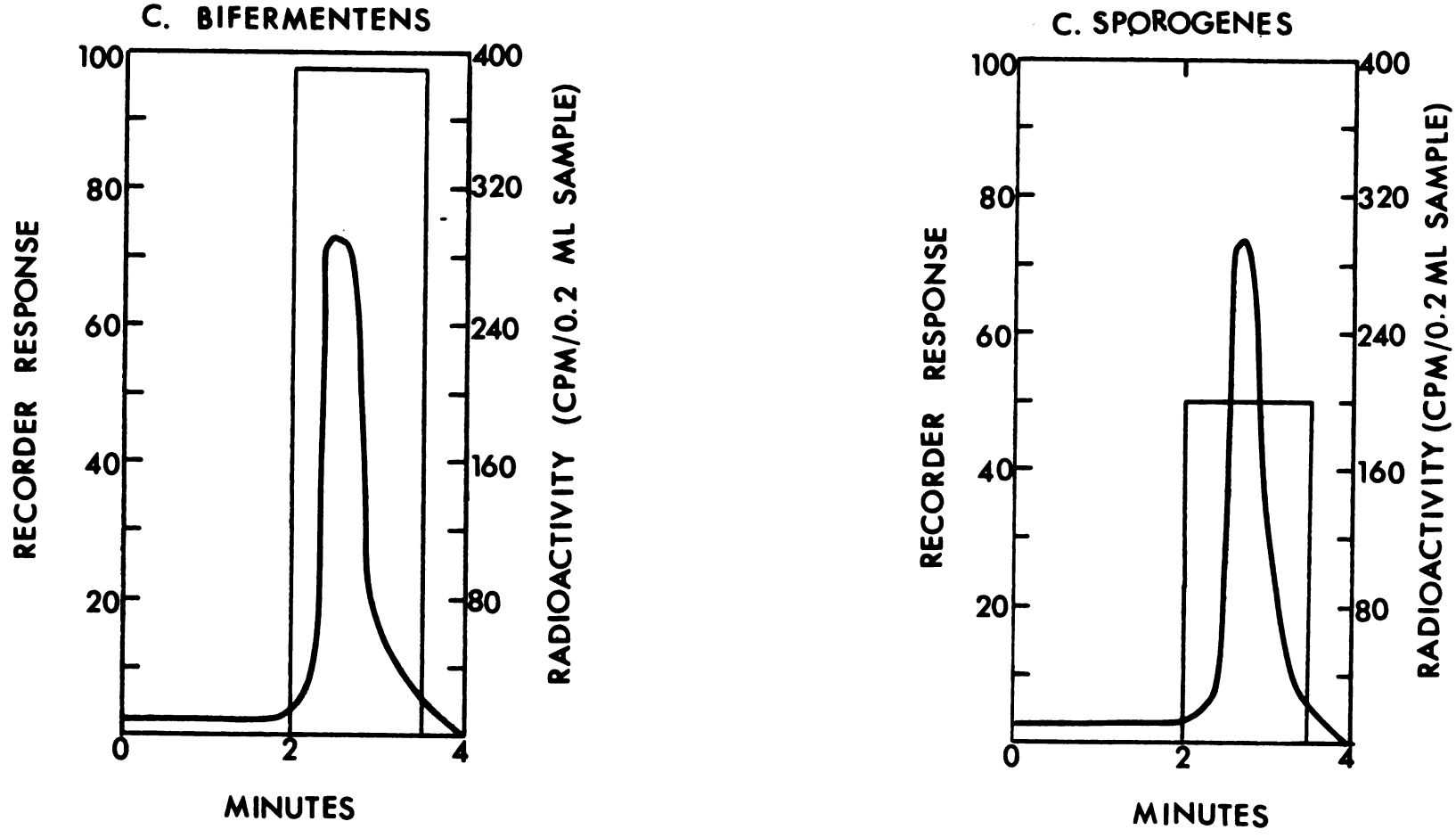


Figure 5. Gas-radiochromatogram of $^{14}CO_2$ produced by clostridial isolates grown in the presence of uniformly labeled ^{14}C amino acid mixture (superimposed upon a chromatographic scan of CO_2 standard).

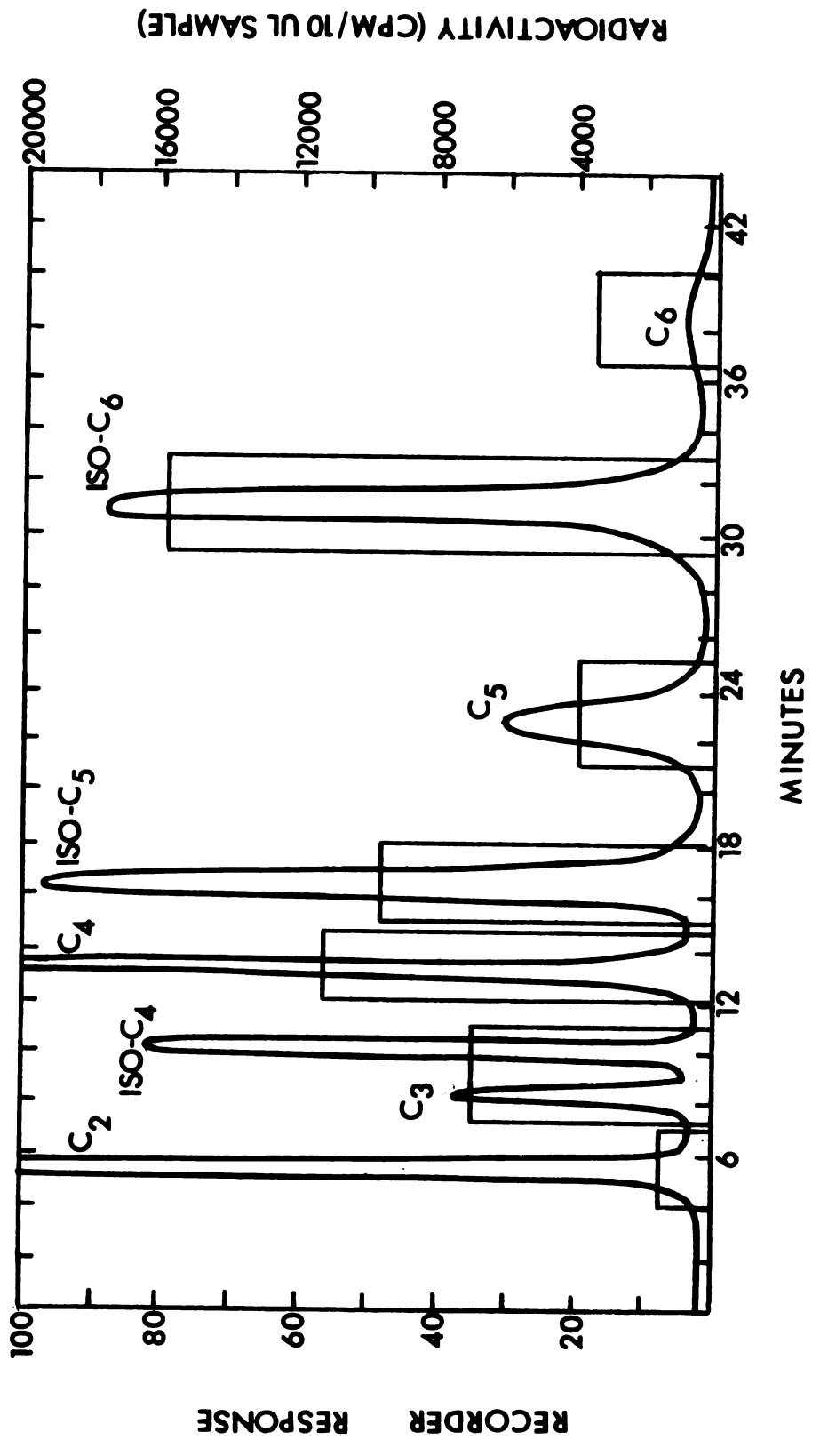


Figure 6. Gas-liquid-radiochromatogram of soluble fatty acids produced by C. bifermentens grown in 1% PY containing 1 µCi/ml ^{14}C (U) -L- amino acid mixture.

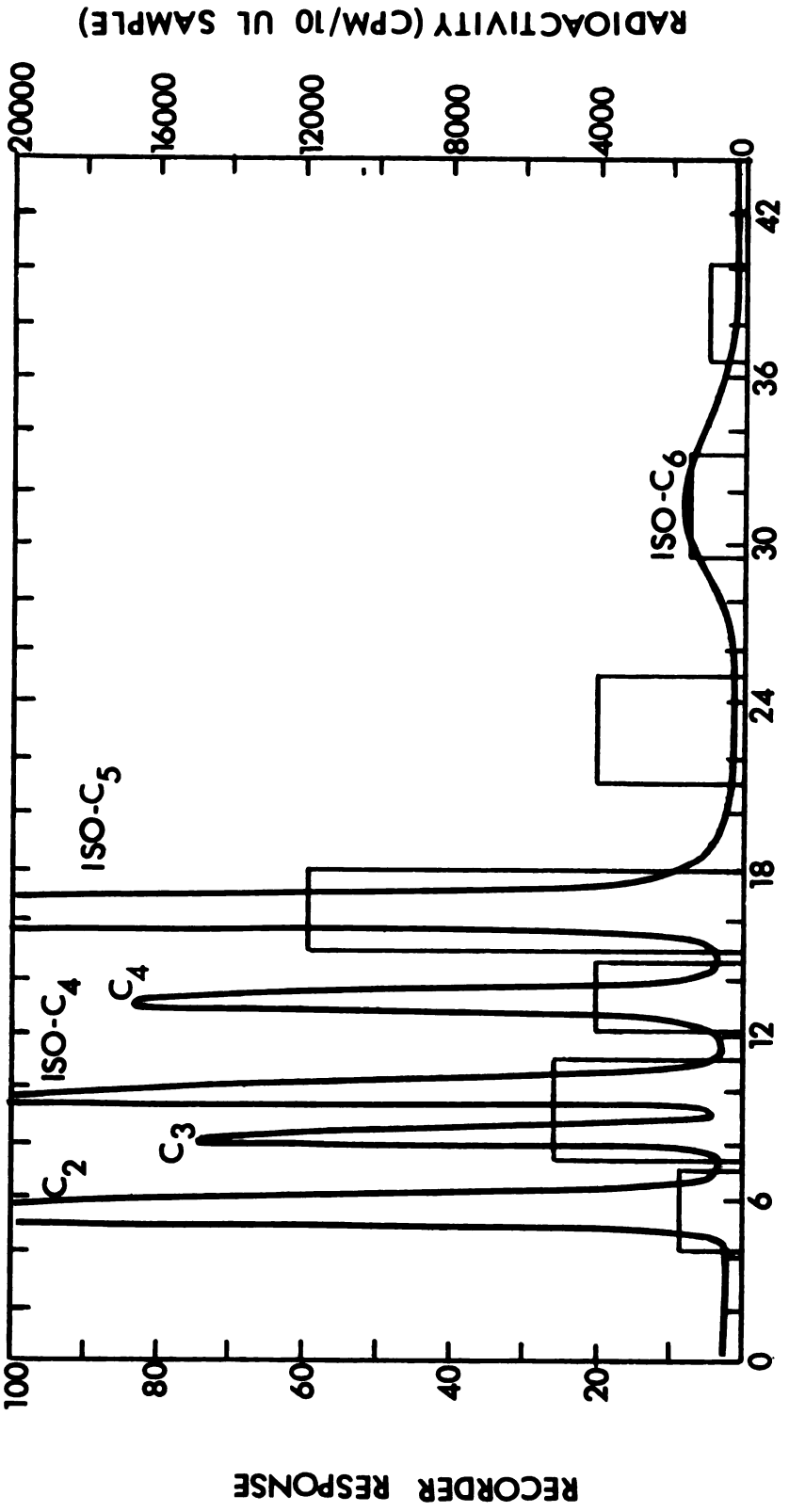


Figure 7. Gas-liquid-radiochromatogram of soluble fatty acids produced by C. sporogenes grown in 1% PY containing 1 µCi/ml ^{14}C (U) -L- amino acid mixture.

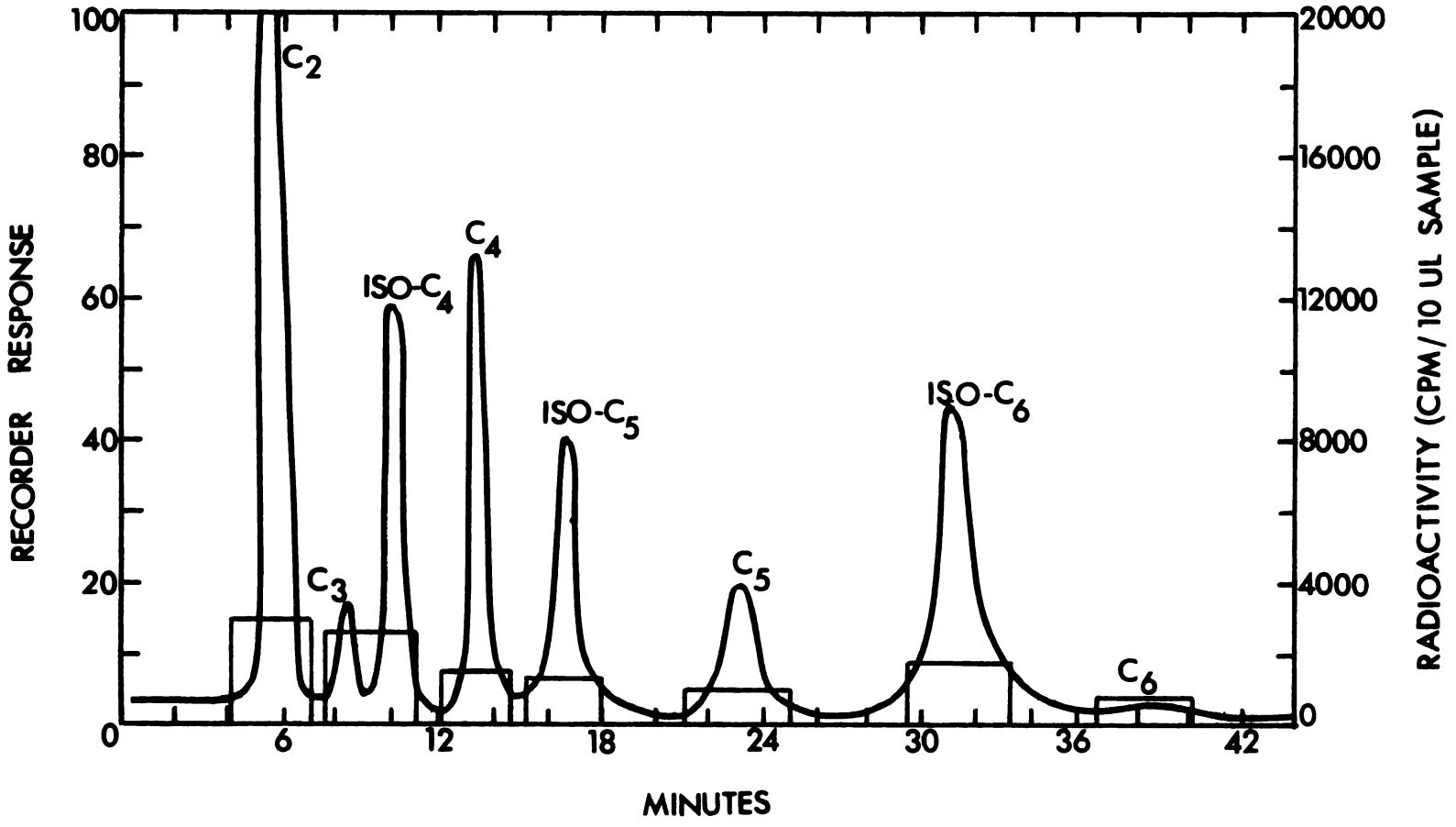


Figure 8. Gas-liquid-radiochromatogram of soluble fatty acids produced by C. bifermentens grown in 1% PYG containing 1 μCi/ml ^{14}C (U) -D-glucose.

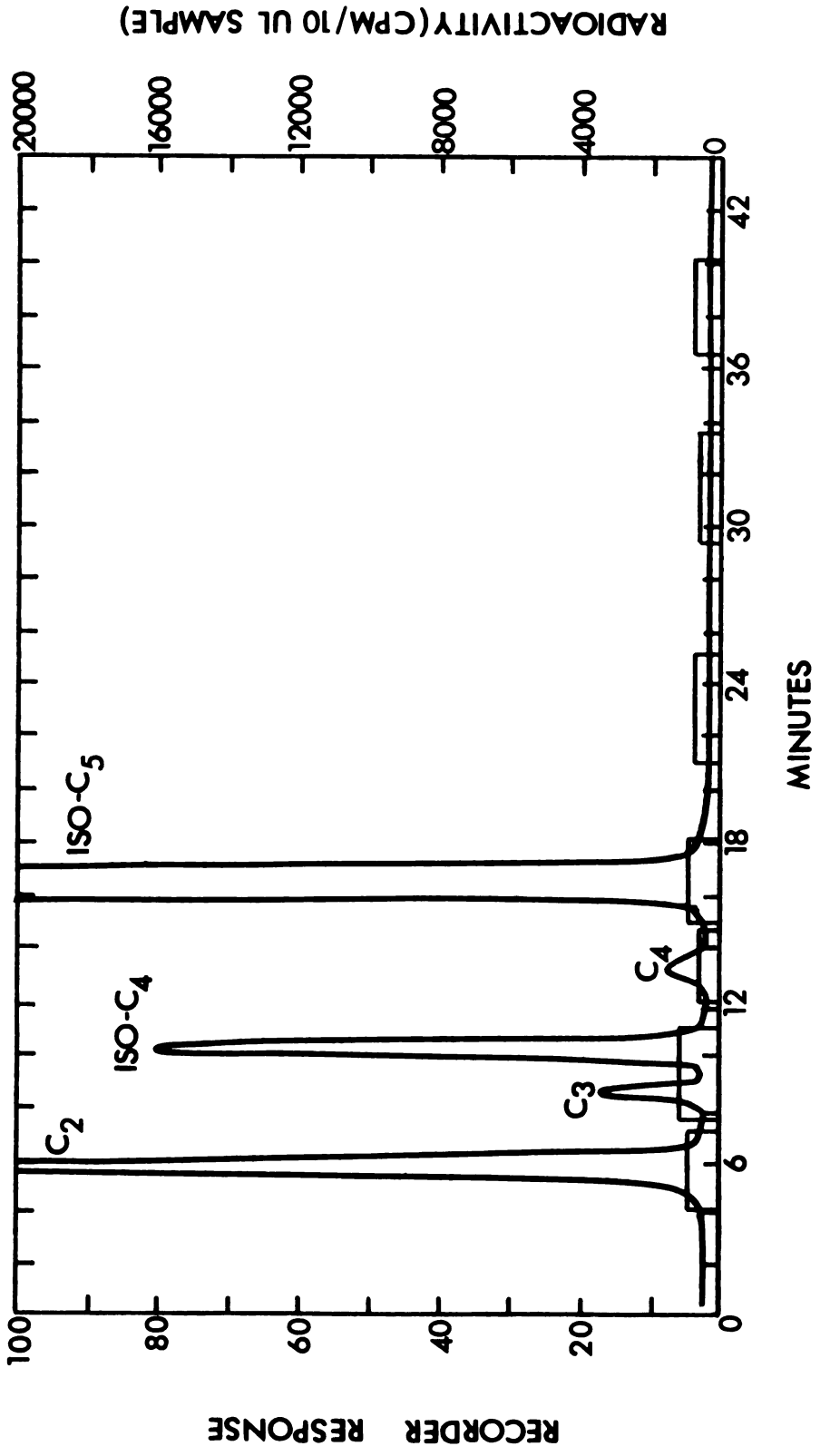


Figure 9. Gas-liquid-radiochromatogram of soluble fatty acids produced by C. sporogenes grown in 1% PYG containing 1 μCi/ml ^{14}C (U) -D-glucose.

caution must be exercised in making interpretations of this type.

Metabolism Of Labeled Organic Compounds By A Natural Sediment Microbial Community

Figures 10 through 12 illustrate the ability of a natural sediment microbial community to convert ^{14}C-amino acids and ^{14}C-glucose to ^{14}C-labeled soluble fatty acids, CO_2, and CH_4 when incubated anaerobically at 10°C. When absolute counts of radioactivity (cpm) alone are considered, more label appears to be incorporated into both the soluble fatty acid fraction and the gaseous fraction when labeled glucose is supplied as substrate as compared to the labeled amino acids. However, as the small quantities of gaseous and soluble metabolic endproducts produced by the sediment microflora were below the sensitivity of both the thermal conductivity and flame ionization detectors under the chromatographic conditions employed, the specific activity of each endproduct cannot be determined. Moreover, the specific activities of the two initial substrates were not the same. Nonetheless, when superimposed upon chromatographic scans of standard mixtures of CO_2 and CH_4, and of a 0.1% standard mixture of fatty acids which were analyzed under the same conditions as the sediment samples, the histograms of radioactivity depicted in Figures 10 through 12 correspond to authentic metabolic endproducts, illustrating the ability of the sediment microbial community to convert labeled organic substrates to

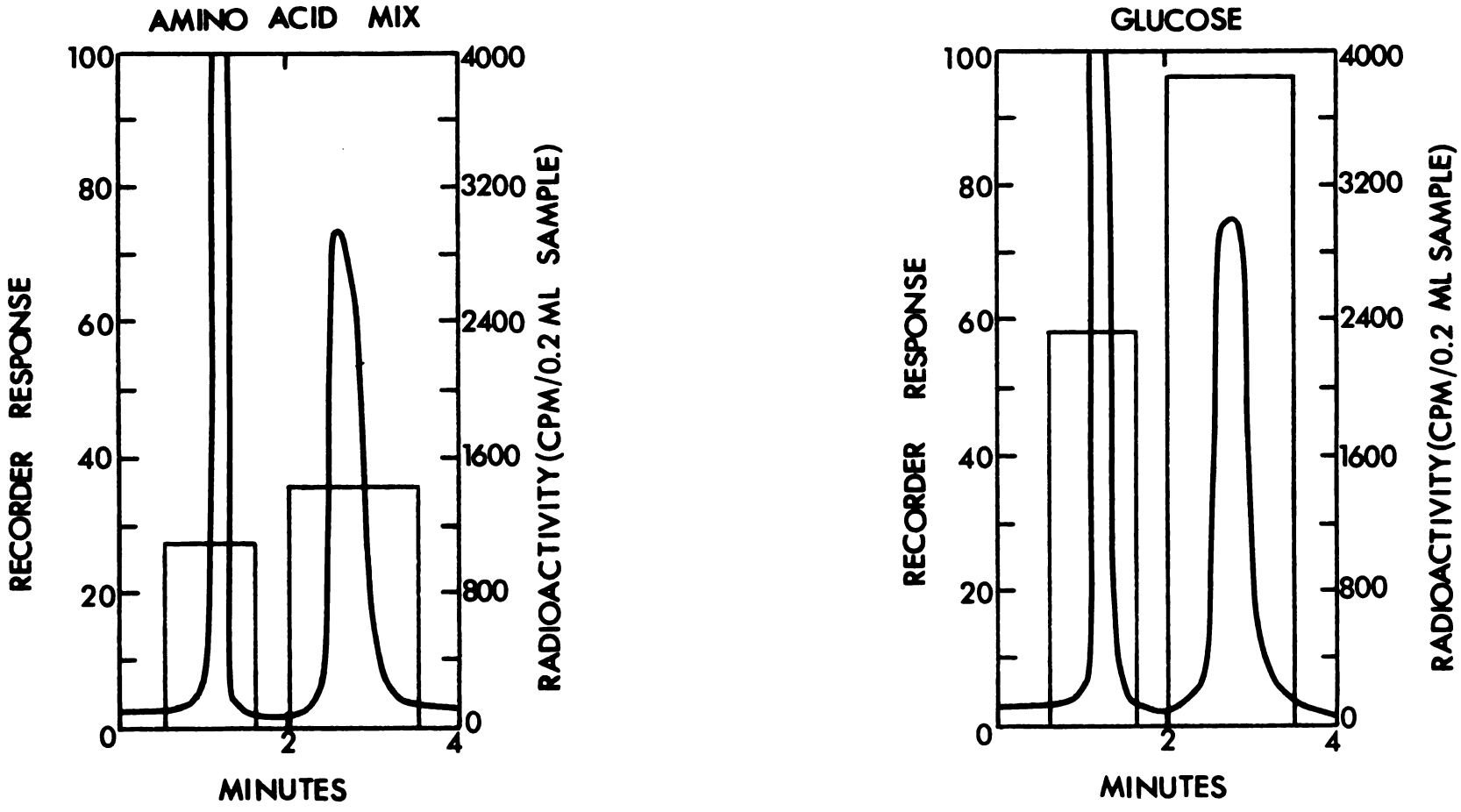


Figure 10. Gas-radiochromatogram of $^{14}CH_4$ and $^{14}CO_2$ produced by sediment microflora incubated in the presence of uniformly labeled ^{14}C amino acid mixture and ^{14}C glucose respectively (superimposed upon a chromatographic scan of a standard mixture of CH_4 and CO_2).

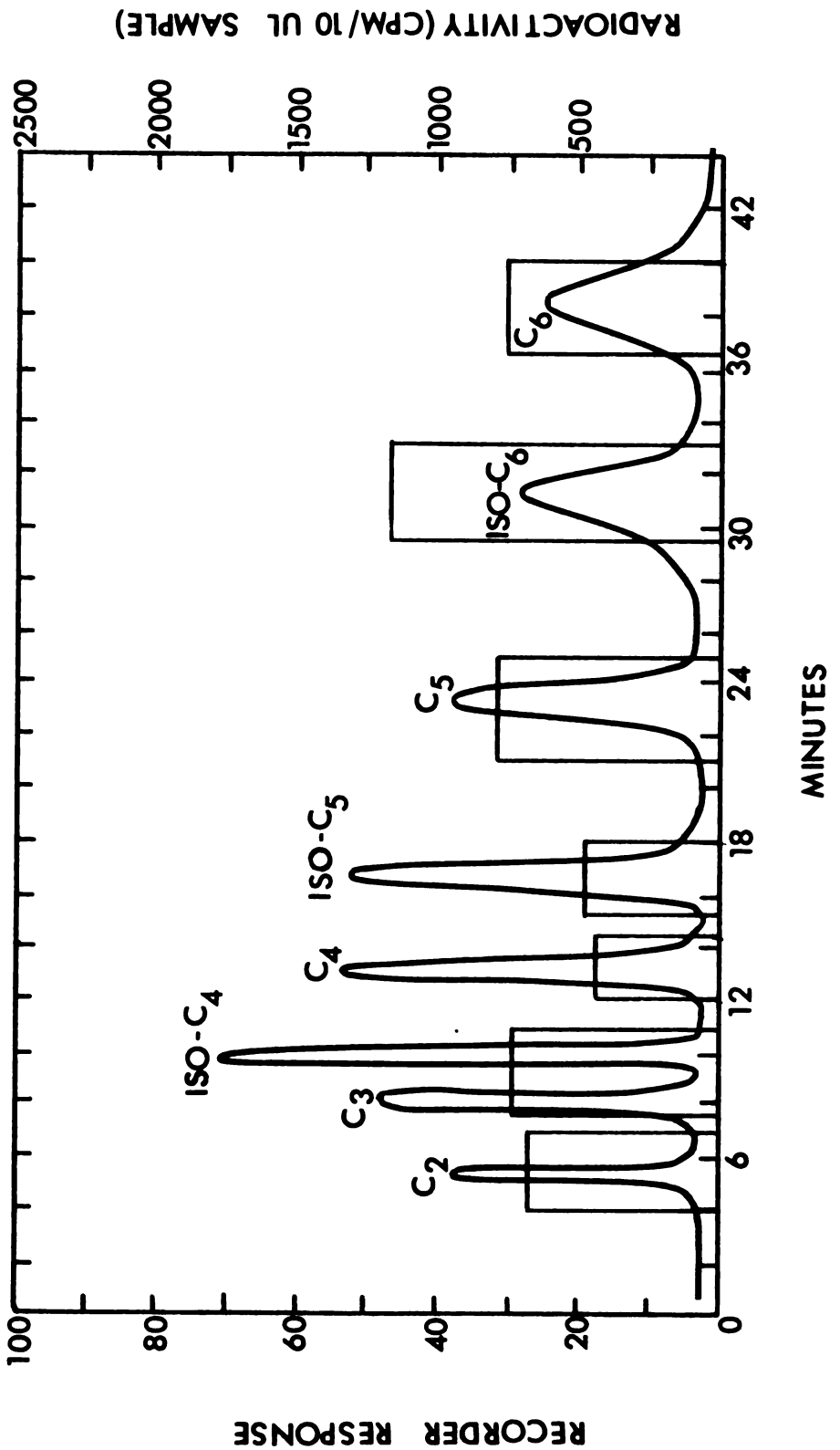


Figure 11. Gas-liquid-radiochromatogram of soluble fatty acids produced by sediment microflora from ^{14}C (U) -L- amino acid mixture (superimposed upon a chromatographic scan of 0.1% standard mixture of short chain fatty acids).

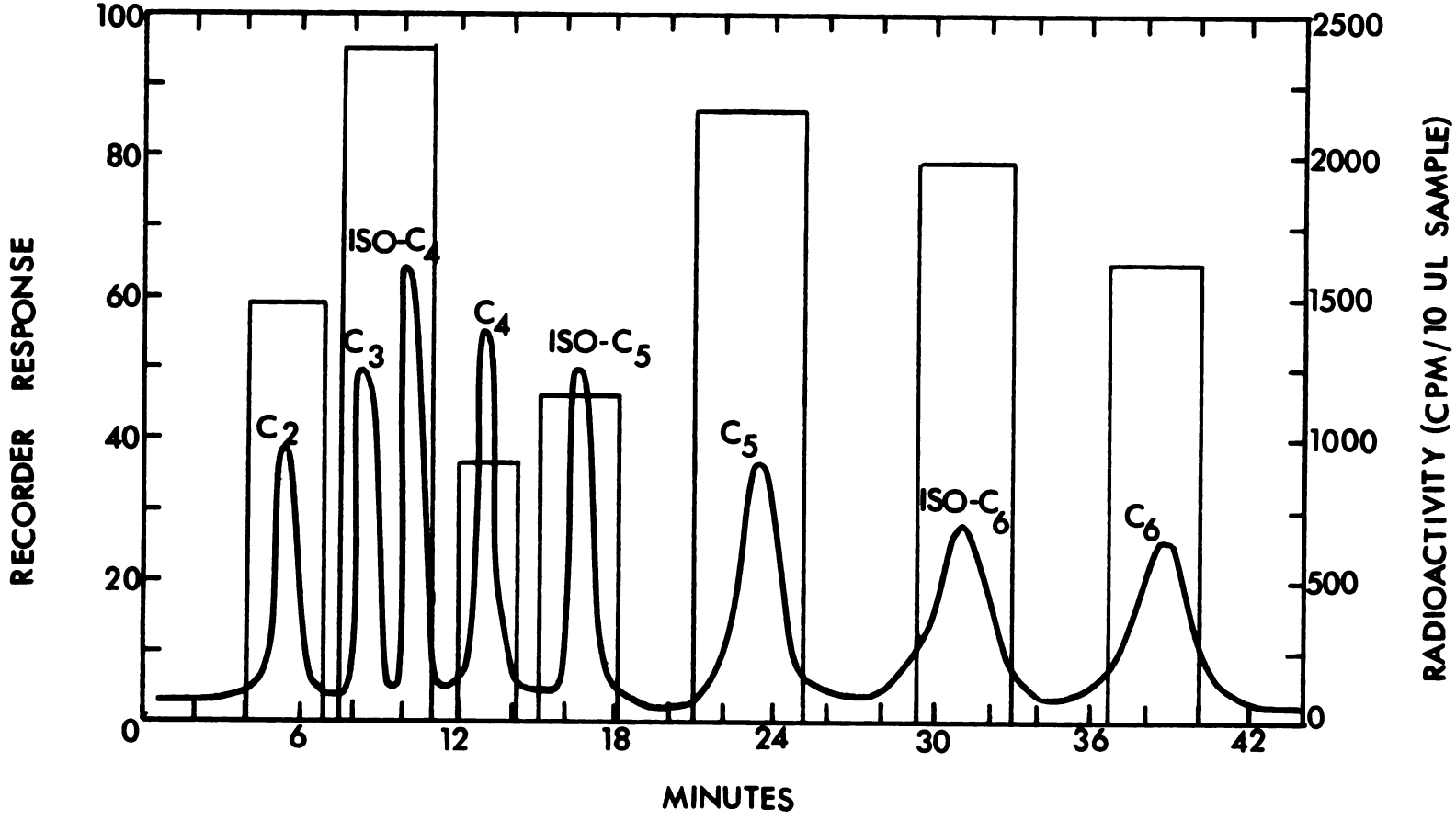


Figure 12. Gas-liquid-radiochromatogram of soluble fatty acids produced by sediment microflora from ^{14}C (U) -D- glucose (superimposed upon a chromatographic scan of 0.1% standard mixture of short chain fatty acids).

gaseous and soluble reduced metabolites under the experimental conditions described.

DISCUSSION

Organic Inputs To The Lake

Provided that the rates of benthic production and permanent loss of carbon to the sediments are known, the rate of flux of carbon from freshwater lake sediments provides a good estimate of overall lake productivity, since benthic metabolism tends to integrate all sources of carbon production (Wetzel and Rich, 1972). Originally applied to studies concerning CO_2 release from the sediments of an oligotrophic marl lake (Rich and Wetzel, 1972), the above generalization can be applied to a hypereutrophic basin such as Wintergreen Lake as well if the flux of carbon from the sediments is expanded to include not only CO_2, but also CH_4, soluble fatty acids and alcohols, and additional forms of dissolved organic carbon released from these sediments. The sediments of Wintergreen Lake are particularly important as a focal point for the accumulation of particulate organic materials. During the study period, the lake received large inputs of allochthonous dissolved and particulate carbon, nitrogen, and phosphorus from migrating and resident waterfowl, as well as from a dairy feedlot effluent. The surface area of the watershed is 7-10 times that of the lake (Manny, 1971). Surface runoff from this large area, which includes a considerable amount of fertilized and cultivated farmland, provides additional inputs of

nutrients to the lake, further fueling the massive algal blooms characteristic of Wintergreen Lake.

These explosive phytoplankton blooms, along with decaying vegetation derived from the extensive littoral macrophyte beds, constitute the primary autochthonous source of particulate organic matter in the lake. Because of the shallow nature of Wintergreen Lake, and the fact that during summer stratification the hypolimnetic oxygen concentration (Figure 3) is insufficient to oxidize the steady influx of organic matter into the basin, much of this material likely reaches the pelagic sediments in a primarily undegraded form. The immediate consequence of this increased accumulation of sedimentary organic matter is a greatly expanded role for the sedimentary anaerobic heterotrophic microflora in the re-cycling of organic compounds in the lake.

Density Of Anaerobic Bacteria At The Sediment-Water Interface

The anaerobic heterotrophic bacterial population present at the sediment-water interface of Wintergreen Lake (Figure 2) showed little variation from March through December, 1973. The mean viable count obtained on pre-reduced PYG plates during this period was 4.24×10^6 organisms/gram dry wt. of sediment. The sediment viable counts reached a maximum in June (Figure 2) corresponding to the onset of significant oxygen depletion in the hypolimnion (Figure 3). However, the counts did not

decline significantly in November and December when the entire water column had become reoxygenated as a consequence of lake turnover. These results likely reflect the near permanent anoxia of these sediments as well as the ability of the large numbers of clostridia present to withstand periodic exposure to oxygenated conditions.

The anaerobic heterotrophic bacterial populations reported in this study are similar to the typical distribution patterns encountered in other lakes of similar trophic level as Wintergreen Lake. Boylen and Brock (1973) reported anaerobic heterotrophic bacterial populations of 4.4×10^5 to 2.8×10^6 organisms/gram wet wt. of sediment in Lake Wingra, Wisconsin during the winter. Surface sediment heterotrophic bacterial densities in the eutrophic lower Great Lakes have consistently ranged between 10^6 and 10^7 organisms/gram dry wt. of sediment regardless of the medium used or the time of sampling (Menon et al., 1971; Vanderpost and Dutka, 1971; Bell and Dutka, 1972a, 1972b; Dutka et al., 1974). Such viable counts represent a very small percentage of the bacterial populations of these sediments. Periodic direct microscopic counts were performed on the sediment-water interface of Wintergreen Lake during the summer of 1974. Organisms were dispersed from the sediment particles by the method of Bohlool and Schmidt (1973) and samples were examined with a fluorescence microscope utilizing acridine orange staining techniques

(Francisco et al., 1973). A mean direct microscopic count of 2.03×10^{11} organisms/gram dry wt. of sediment was obtained, a value not unrealistic for organically enriched habitats.

Heterotrophic bacteria are present wherever sufficient organic matter is present, in numbers that are usually proportional to the quantity of organic matter available (Dutka et al., 1974). However, as organic nutrients are generally present in greater than optimal quantities in eutrophic lake sediments, heterotrophic bacterial communities are limited by the ability of their members to survive and metabolize in a deoxygenated environment rather than by the organic content of the habitat. Consequently, once a stable bacterial community is established in these sediments, the continued addition of organic material to the system such as occurs in Wintergreen Lake may not necessarily result in an increased bacterial biomass (Dutka et al., 1974). Rather, the sudden and dramatic crashes of phytoplankton blooms observed in Wintergreen Lake, similar to the massive algal rains documented in Lake Erie (Burn and Boss, 1972), may, while providing additional nutrients to the sediments, also tend to bury the endemic population. Only the surface bacterial population would be actively involved in the degradation of this added nutritive material, thus explaining the relatively stable bacterial community and steady build-up of organic materials in the sediments of

Wintergreen Lake and other eutrophic basins (Dutka et al., 1974).

Anaerobic Bacteria Isolated From The Sediment-Water Interface

The most striking feature of the bacterial groups isolated from Wintergreen Lake sediments was the high predominance of clostridia among the organisms examined. The genus Clostridium accounted for 71.8% of the total isolates (Table 5). Greater than 50% of the clostridial strains in turn were identified as Clostridium bifermentens and C. sporogenes (47.7% and 17.8% respectively).

Although clostridia are generally held to be widely distributed in nature (Stanier et al., 1970; Slepecky, 1972; Doetsch and Cook, 1973), studies of their occurrence in freshwater and marine sediments have, for the most part, been limited to considerations of these habitats as reservoirs of infection for spores of clostridia pathogenic to humans and animals (Laycock and Longard, 1972; Laycock and Loring, 1972; Sugiyama et al., 1972). Smith (1968), working with marine sediments, compared the clostridial flora of a productive sediment and an organically deficient sediment. On the basis of preliminary data, he suggested that (1) proteolytic clostridia such as C. sporogenes, C. bifermentens, and C. sticklandii may be essential members of most marine sediment microbial communities regardless of their organic content; (2) that primarily saccharolytic clostridia can

sustain themselves in a sedimentary habitat only when the latter is organically enriched and constantly replenished with nutrients, and (3) that more species of clostridia may be present in environments with a high organic content.

Matches and Liston (1974) examined the sediments of Puget Sound for the presence of clostridia. They reported anaerobic plate counts ranging from 0.73 to 23.5 x 10^4 cells/ml of sediment-water slurry. Approximately 30% of the organisms obtained were determined to be clostridia, the three species isolated in greatest numbers being C. perfringens, C. bifermentens, and C. novyi. As the sediment temperature of Puget Sound rarely rises above $10^{\circ}C$, which has been reported to be the minimum temperature for the germination and growth of most clostridia (Matches and Liston, 1974), they concluded that the resident sedimentary population of these organisms was derived from terrestrial sources rather than from active growth within the sediments.

The highly proteolytic nature of most of the clostridial isolates was particularly striking (Table 7). Although the sediments of Wintergreen Lake are organically enriched, very few saccharolytic clostridial strains were found, the single saccharolytic representative, C. butyricum comprising only 7% of the total isolates. The diversity of the clostridial isolates was particularly low as well (Table 5). As clostridial taxonomy has

traditionally been problematical, the performance of additional definitive biochemical tests might demonstrate the presence of other species of clostridia among the isolates examined. Enrichments for specific physiological types of clostridia such as cellulose degraders, while not performed in the present study, might also reveal the presence of additional members of this genus in Wintergreen sediments.

The ability of most clostridia to grow at 10°C (Matches and Liston, 1974) should impart these organisms with a favorable selective advantage over other species of strictly anaerobic bacteria by allowing them to grow and metabolize in cold environments. Such appears to be the case in Wintergreen Lake. The temperature of the sediment-water interface remained at 10°C or greater for nearly 6 months of 1973, reaching a yearly high of 13.3°C in October (Figure 4). This upper range of sediment temperature correlates well with the period of most extensive oxygen depletion in the hypolimnion (Figure 3) and with the peak in anaerobic heterotrophic bacteria (Figure 2), indicating that conditions during the summer months are optimal for the growth of the clostridia.

The three clostridial isolates obtained from Wintergreen Lake were able to grow at 10°C (Table 6). Thus, while additional clostridial inocula were supplied to Wintergreen sediments from terrestrial sources and from fecal contamination from aquatic waterfowl, a

resident sedimentary community was metabolizing and growing *in situ*, particularly during periods in the summer and early fall when sediment temperature and anoxic conditions in the hypolimnion were most favorable for growth. The ability of these organisms to form spores would then presumably allow them to withstand periods unfavorable for growth, as during lake turnover, or during periods of reduced sediment temperature (Doetsch and Cook, 1973).

Although isolated in far fewer numbers than the clostridia, the facultative cocci of group I and the anaerobic *Vibrio* of group II (Table 5) are probably active members of the natural sediment community. Each demonstrated the ability to grow at environmental temperatures (Table 6). The group I *Streptococcus* failed to hydrolyze any of the complex substrates tested (Table 7), but vigorously fermented all of the sugars tested (Table 6). Presumably, this organism thrives in the sediments by metabolizing the soluble carbohydrates present or the breakdown products resulting from the attack of other bacteria on more complex substrates. Unlike the *Streptococcus*, the anaerobic *Vibrio* may actively be involved in the degradation of particulate material in the sediments. This organism, like the clostridia, was primarily proteolytic (Table 7) rather than saccharolytic (Table 6).

Considered as a whole, the Wintergreen sedimentary isolates are characterized by a high percentage of obligate anaerobes as compared to the number of facultative bacteria obtained and by the strikingly limited hydrolytic capabilities of these facultative organisms. The greater hydrolytic abilities of the strictly anaerobic isolates, particularly their proteolytic capabilities, could provide these organisms with a selective advantage in an organically enriched environment such as the sediments of Wintergreen Lake, where a predominant portion of the organic matter present is in a particulate or complex form.

Anaerobic Metabolism of Labeled Organic Substrates By Clostridial Isolates and By Sediment Microbial Communities

The genus Clostridium includes a large number of species capable of utilizing widely differing catabolic pathways for decomposition of proteins, carbohydrates, and additional complex substrates (Barker, 1961; Wood, 1961; Stanier et al., 1970; Mead, 1971). Moreover, these bacteria have been demonstrated to produce a wide array of metabolic endproducts depending upon the medium, cultural conditions, and analytical techniques employed (Stanier, 1970; Farshy and Moss, 1970; Anema et al., 1973). Due to these complexities, many of the details of the diverse catabolic capabilities of these organisms remain unresolved (Barker, 1961).

The catabolism of amino acids is carried out by a great number of obligate and facultative anaerobic bacteria, including numerous clostridia (Doelle, 1969). Although many clostridia ferment single amino acids (Barker, 1961; Doelle, 1969), the two isolates dealt with in the present study, C. bifermentens and C. sporogenes, obtain most of their energy by a coupled oxidation-reduction reaction between suitable amino acids or between amino acids and a non-nitrogenous compound. The coupled decomposition of amino acids is referred to as the Strickland reaction (Barker, 1961). The characteristic feature of this reaction is that while single amino acids are not decomposed appreciably, appropriate pairs are degraded rapidly. One member of the pair is oxidized while the other is reduced (Barker, 1961).

The volatile short-chain fatty acids produced by C. bifermentens and C. sporogenes in PYG broth (Table 5) are characteristic for these organisms (Moore et al., 1966; Holdeman and Moore, 1972). With the exception of a few species of anaerobic cocci (Peptostreptoccus sp.) and a small number of Bacteroides sp. and Eubacterium sp., the production of valeric and iso-valeric acids as fermentation endproducts is generally limited to the genus Clostridium (Moore et al., 1966). Furthermore, production of major quantities of caproic and iso-caproic acids as fermentative endproducts is almost exclusively restricted

to the clostridia (Barker, 1961; Wood, 1961; Holdeman and Moore, 1972). A pertinent consequence of these observations is that the production of significant amounts of these fatty acids by a natural sediment microbial community would provide strong presumptive evidence for the active role of clostridia in the decomposition of organic substrates in these sediments.

Gas-liquid-radiochromatographic experiments were performed to test this hypothesis in the sediments of Wintergreen Lake (Figures 10 through 12). Although the specific activity of each individual labeled metabolite could not be determined, it is nonetheless clear that significant quantities of radioactivity were detected at time intervals corresponding to the chromatographic retention times of a series of soluble fatty acids (acetic through caproic) after incubation of a sediment microbial community at 10°C in the presence of labeled organic substrates (Figures 11 and 12). Of particular significance is the incorporation of radioactivity in those regions corresponding to the valeric and caproic series of fatty acids respectively, when either ^{14}C-labeled glucose or amino acids were utilized as the initial substrate. The incorporation of radioactivity into these "marker" fatty acids provides strong presumptive evidence that clostridia, and possibly C. bifermentens and C. sporogenes in particular (Figures 6 and 7), are active in the degradation of organic substrates in

Wintergreen Lake sediments.

Similarly, an analysis of the gaseous metabolites produced by the natural sediment microflora after anaerobic incubation at 10°C (Figure 10) demonstrated the production of $^{14}CH_4$ and $^{14}CO_2$, the collected radioactive effluents corresponding well with the retention times of CH_4 and CO_2 standards analyzed under the same conditions. The significance of this finding is that it points out the role played by anaerobic heterotrophic bacteria, such as the clostridia in the production of soluble and gaseous metabolites which can serve as precursors for further metabolism by additional groups of lake bacteria, such as the methanogenic bacteria. For instance, the vigorous production of H_2 by the clostridia (Table 6; Barker, 1961; Gray and Gest, 1965) assumes added importance in light of the recent assertion that the methanogenic bacteria of lake sediments are autotrophs and utilize CO_2 and H_2 in the formation of methane (Nelson and Zeikus, 1974).

The decomposition and recycling of organic carbon in the pelagic zone of Wintergreen Lake is undoubtedly controlled by primarily anaerobic processes in the sediments which result in the production and release of significant quantities of reduced dissolved organic matter and gaseous metabolites. Although not specifically considered in the present study, decomposition in the littoral zone of the lake is likely controlled by

similar processes as well. This investigation has demonstrated the predominance of clostridia among the isolatable strictly anaerobic bacteria of the pelagic sediments of a eutrophic hardwater lake, and has presented strong presumptive evidence that these clostridial species are active in the decomposition of organic matter present in these sediments.

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