METHANE CYCLING IN LAKE 227
AND ITS EFFECTS ON WHOLE LAKE METABOLISM

A Thesis

Submitted to

the Faculty of Graduate Studies

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

by

John William McCullagh Rudd

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TO LESLEY AND MY FAMILY

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The Four Stumbling Blocks to Truth

1. The influence of fragile or unworthy authority.

2. Custom.

3. The imperfection of our undisciplined senses.

4. Concealment of ignorance by ostentation of seeming wisdom.

Roger Bacon, English Philosopher, Magnus, and man of science

1220 - 1292

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GENERAL INTRODUCTION

The research for this thesis was mainly performed at the Experimental Lakes Area (ELA), in northwestern Ontario (Johnson and Vallentyne, 1971). The ELA facility was established in response to an interim report on eutrophication in the lower Great Lakes issued in December of 1965 by the International Joint Commission. Initially the main emphases of research were experimental eutrophication studies on several small lakes which were artificially eutrophied by addition of various combinations of phosphorus, nitrogen and carbon. The intentions were to quantitatively define the causes of the eutrophication problem, to determine its overall ecological effects and to devise remedial methods.

These intentions were simplified by two of the advantages of the ELA facility. The research camp was remote yet accessible. Thus the whole lake experiments could be performed in the absence of uncontrollable cultural influences. Also, effects of ground water influences were minimal since the experimental lake basins were composed of impervious Precambrian igneous rock. For these reasons accurate estimates of nutrient loading rates (both natural and artificial) could be obtained enabling the quantitative whole lake approach to the study of the eutrophication problem.

Commencing in 1969 Lake 227 was artificially eutrophied by weekly additions during the ice free season of phosphorus and nitrogen (0.48 gP/m^2 and 6.29 $gN/m^2/yr$) (Schindler et al. 1971 and 1973). The lake responded quickly to the increased nutrient loading rate and within five years exhibited many of the classical characteristics of

a highly eutrophic lake. During summer, epilimnetic pH and chlorophyll concentrations increased while secchi depth decreased (Schindler et al. 1973). During summer, as a result of the decomposition of the larger quantities of phytoplankton biomass the hypolimnion was depleted of dissolved oxygen more rapidly and completely (Schindler et al. 1973). As predictedby Schindler et al. (1973) the year-to-year trend of progressively less dissolved oxygen during winter culminated in the development of total lake anoxia during the winter of 1973-74 resulting in suffication of many fishes, zooplankton and zoobenthos.

Another consequence of this intense eutrophication was the accumulation of large quantities of dissolved methane in the anoxic hypolimnion during periods of summer and winter stratification. The purpose of this study was to determine how much of the photosynthetically fixed carbon was regenerated as methane and what proportion of this methane either degassed to the atmosphere or was recycled within the lake as carbon dioxide and bacterial cell material by methane oxidizing bacteria. A second objective was to define what effects methane cycling (ie. the bacterial production and oxidation of methane) had on whole lake metabolism.

For two reasons its became obvious very early in the study that it was most important to be able to accurately monitor the methane oxidation step of the methane cycle. This step has a large potential for effecting whole lake metabolism since the oxidation of methane consumed dissolved oxygen, produces carbon dioxide (a carbon source for primary production) and produces bacterial cell material (an alternative fixed carbon for secondary grazers). Secondly, if the rate of methane oxidation at the oxic - anoxic interface in the water column could be accurately

monitored a methane production rate for the entire hypolimnion could be estimated by summing the methane oxidized at the oxic - anoxic interface and the increase in the total mass of dissolved methane in the anoxic hypolimnion during periods of stratification. Therefore the methane oxidation process was emphasized in this research project since it was the key to illucidating whole lake effects of methane cycling.

HISTORICAL

The escape of combustible gases from sources in the earth's crust was first noted in Roman times by Pliny (Barker, 1956). However Volta (Söhngen, 1906) is generally credited with the discovery of methane. In 1776 he described the formation of "combustible air" in the sediments of several lakes, ponds and streams. The gas was not characterized however until 1806 by William Henry.

Methane Production

In 1868, Bêchamp (quoted in Barker, 1957), first suggested that methane had a biological origin. He described a fermentation in which ethyl alcohol and calcium carbonate was converted to large amounts of methane, as well as some carbon dioxide and fatty acids. Söhngen (1906) described two organisms that produced methane from the lower fatty acids. Although he believed these to be pure cultures of methane producing bacteria, it wasn't until 50 years later than pure cultures of two methane producing bacteria were actually obtained by Schnellan (quoted in Stadtman 1967).

The availability of pure cultures enabled research into the biochemistry of methanogenesis which has been reviewed recently by Wolfe (1971). Progress in this line of research was complicated by the culture Methanobacterium omelianskii (Barker, 1940) which was thought to be a pure culture converting ethanol to methane and acetate. Instead Bryant et al. (1967) discovered that it was a mixed culture consisting of two different bacteria, one converting ethanol to acetate and molecular hydrogen the other reducing carbon dioxide to methane using

the hydrogen produced by the other symbiont. As a result many of the earlier studies of methanogenesis are now difficult to interpret (eg. Stadtman and Barker, 1949 Barker, 1956, Stadtman, 1967, Knight et al. 1966). The resolution of this culture also emphasized the simplicity of substrates that methane producing bacteria are capable of using. It is now believed that only four compounds, carbon dioxide, acetate, methanol, and formate are converted to methane by the methane producers (Wolfe 1971). The much more complicated pathways of methane production originally attributed to these bacteria (eg. Stadtman 1967) are thought to be erroneous.

Thus the true role of methane producing bacteria in anaerobic degradation appears to have emerged. Anaerobic biodegradation (whether it be in sewage digesters, the rumen or in sediments) is thought to be a three stage process (Cappenberg 1975, Toerien and Hattingh 1969). The first non-methanogenic stage is composed mainly of various obligate anaerobic bacteria which are believed to be important in the conversion of complex proteins, fats and carbohydrates to much simpler compounds such as carbon dioxide, organic acids and molecular hydrogen. In the second also non-methanogenic stage the organic acids are converted to the organic substrates of the methane producing bacteria (such as acetate, methanol and formate). The sulphate reducers which convert lactate to acetate (Cappenberg 1975) are likely important members of this group. The third stage comprise the methanogenic bacteria. Most of the methane production is believed to originate from two substrates; acetate which is cleaved to yield a molecule of methane and carbon dioxide and carbon

dioxide which is reduced using molecular hydrogen. This hydrogen oxidation is considered to "pull" the degradation of the anaerobic microbial food chains by displacing unfavourable equilibria (Wolfe 1971). The methane and carbon dioxide produced during methanogenesis is non-toxic so that the end products of this balanced anaerobic biodegradation can accumulate to very high concentrations without causing inhibition of the overall process.

Measurement of fresh water and marine methane concentrations in sediments and the water column have been extensively reported over the last twenty years (Koyama 1953, 1964, Reeburgh and Heggie 1974, Reeburgh 1976, Cappenberg 1975, Weimer and Lee 1973, Atkinson and Richards 1967, Lamontagne et al. 1973, Deuser et al. 1973, and Martens 1974). However the study of the methane production process and rates of methane production in sediments began only recently (although sediments had been used previously as innocula for the isolation of methanogens). Which one of the two principle substrates of methanogens (acetate and carbon dioxide) is most important remains undecided. Claypool and Kaplan (1974) used the changes in the C^{13} content to suggest that 30-50% of the methane production occurred as a result of carbon dioxide reduction. Deuser et al. (1973) convincingly contended that volcanic carbon dioxide and hydrogen was used by methanogenic bacteria to produce the very high methane concentrations found in lake Kivu (20 m moles/liter). Also, Nelson and Zeikus (1974) reported that hydrogen stimulated methanogenesis in sediments while acetate did not. They concluded that carbon dioxide reduction was the major methane source in sediments. However Cappenberg (1975) concluded that approximately 70%

of the methane production occurring in Lake Vechten originated from and that sulphate reducers had a commensal relationship with the methane producers. Using specific inhibitors he showed that the sulphate reducers converted lactate to acetate and assisted in reducing the Eh to the low level required by the methanogens (<250 mv). The methane producers used the acetate as a substrate, splitting it into methane and carbon dioxide. Thus the relative importance of carbon dioxide and acetate as precursors for methane production in aquatic environments varies from one body of water to another and will probably be dependent upon the availability of carbon dioxide and hydrogen in one case and acetate in the other case.

Cappenberg (1975) also found that the sulphide produced by the sulphate reducers was inhibitory to methane production at $pS^{=}$ values below 10.5. This appeared to control the distribution of methanogenic bacteria and they were confined to a zone of low sulphide concentration below the zone of active sulphide production. However Martens and Berner (1974) had a different point of view. They contended that the sulphate present in the zone of sulphide production in shallow marine sediments was responsible for the inhibition of methanogenesis and consequently the methanogens were confined to a zone deeper in the sediments where sulphate concentrations were lower.

Attempts have been made to measure rates of methane production in sediments (eg. Mallard and Frea 1972, MacGregor and Keeney 1973, and Koyama 1962). However the in vitro approaches used by these authors seriously limited the utility of the data since the bottle incubation of disturbed sediments isolated from their natural environment would probably alter methane production rates in an unknown way. Oremland

(1975) used an in vivo method to measure methane production rates in a variety of shallow marine sediments. He trapped methane in chambers as it diffused away from the sediments and estimated that production rates varied from 0.05 -45 Kg/hectare/yr. Representative measurements of methane production rates in other aquatic environments are lacking.

Methane Oxidation

In 1905 Käserer (quoted in Wake et al. 1973) reported the existence of methane oxidizing organisms in soil. However Söhngen (Wake et al. 1973) first isolated an organism in 1906 which was capable of utilizing methane as its sole source of carbon and energy. Doubts have since been expressed concerning this culture's purity (Dworkin and Foster 1956). This same culture has apparently been reisolated and/or renamed three times in the succeeding 64 years and is presently known as Methylomonas methanica (Whittenbury 1970).

Five types of methane oxidizing bacteria have recently been described (Whittenbury 1970). These bacteria utilize only one-carbon compounds, are gram negative and strictly aerobic. They possess one of two types of internal membrane systems which are thought to be involved in the methane oxidation process. The biochemistry of the methane oxidation process has been reviewed by Foster (1961), Kosaric and Zajic (1974), Quayle (1961), Ribbons et al. (1970) and Wilkinson (1971).

For a time it was believed that these five types were the only organisms capable of oxidizing methane (reports of mycobacteria and eucaryotes oxidizing methane appear to be unsubstantiated). However Reeburgh (1976) and Reeburgh and Heggie (in press) have presented evidence that anaerobic oxidation of methane by sulphate reducing bacteria

(which was originally reported by Davis and Yarbrough, 1966) may be an important factor in the methane cycle of the oceans. Also, Patt et al. (1976) have reported the isolation of a methane oxidizing bacterium which is capable of utilizing a variety of organic substrates in addition to methane as a sole source of carbon and energy.

Methane oxidizing bacteria also appear to be active in the cycling of nitrogen. Hutton and Zobell (1953) reported that methane oxidizing bacteria oxidized ammonia to nitrite, while Davies (1973) claims to have isolated methane oxidizers which were capable of utilizing methane as a hydrogen donor for denitrification. However this use of nitrate as an electron acceptor is in direct disagreement with the conclusion of Whittenbury et al. (1970). It appears certain that nitrogen fixation is a fairly common property of methane oxidizing bacteria (Davis et al. 1964, deBont and Mulder 1974, and Whittenbury et al. 1975).

Although the presence of methane oxidizing bacteria in both fresh-water and marine environments has been known for many years (Söhngen 1906 quoted in Wake et al. 1973 and Hutton and Zobell 1949), very little work has been done on the role of these bacteria in the aquatic environment. Numbers of methane oxidizing bacteria in the water column and at the sediment water interface have been estimated (Cappenberg, 1975 and Whittenbury et al. 1975). This approach however gives no information about in situ rate of methane oxidation and thus reveals little about the role of methane oxidizers in carbon cycling in aquatic ecosystems. Howard et al. (1971) attempted to estimate the importance of methane oxidizers in carbon cycling of Lake Erie but their methods of measurement of rates of methane oxidation were deficient in that they

increased the in situ methane concentrations of the water samples before incubation began and then incubated for a very long time (5 days). Weaver and Dugan (1972) demonstrated that clay particulate material enhanced rates of methane oxidation in enrichment cultures of methane oxidizing bacteria obtained from Lake Erie. However the possible importance of this in nature was not established. Very few reliable rates of methane oxidation have been reported. Jannasch (1975) estimated that methane was oxidized at the oxic-anoxic interface of Lake Kivu at up to 0.01 μmoles/L/hr. Belyaev et al. (1975) reported that methane was oxidized in the Mari Lakes at rates varying from 0.01 to 1.2 μmoles/L/hr. No reliable estimates of the importance of methane oxidizers to carbon cycling or to consumption of dissolved oxygen in lake water is available.

GENERAL METHODS

I Measurement of in situ rates of methane oxidation

Sampling

Lake 227 water samples were pumped from a point source using thick walled 6 mm I.D. Tygon tubing attached to a peristaltic pump. The sample tubing was lowered to depth by the progressive addition of meter or half meter lengths, taking care to eliminate coils in the tubing at the surface where degassing could occur. If the sampling boat was anchored securely at bow and stern it was determined that the point source could be maintained at a precise depth by use of a styrofoam float. Samples were routinely obtained from 0.25 or 0.1 meter intervals.

Three samples from each depth were pumped into 125 ml pyrex reagent bottles. At least twice the volume of the bottles was displaced in order to minimize invasion of atmospheric oxygen. The sample bottles were immediately sealed with glass stoppers whose tips had been reground to a conical profile. One of the three samples was killed in the field by injecting NaOH to produce pH 11. An extra, unfixed subsample was taken from the sample representing the anticipated depth of maximum activity. All samples were transported to the laboratory (0.75 hr) in light-tight containers at temperatures within 2°C of *in situ* temperatures.

Analyses

In situ methane concentration:

Twenty-five milliliters of each sample fixed at the lake, followed by 25 cc of helium, were drawn into a 50-cc disposable plastic syringe fitted with an 18-G 2.5 cm needle. This needle was replaced with a 26-G needle, the tip of which was inserted partially into a rubber bung

to provide a temporary seal. Thirty seconds of vigorous shaking was sufficient to strip 97% of the dissolved CH_4 from the sample. The gas phase was then injected into a capped inverted 6-ml serum bottle filled with distilled water displacing the water through a second 26-G needle. The excess gas phase was used to flush the serum bottle after all the distilled water had been expelled. This procedure permitted replicate analyses of a sample as well as sample storage for up to 1 week. Two-tenths of a milliliter of this sample was injected into a Pye 104 gas chromatograph equipped with a flame ionization detector and a phenyl isocyanate/Porasil C column. In situ methane concentrations were calculated from peak height and expressed as μmoles methane per liter (μM) of lake water.

Effects of the introduction of a gas phase into a water-filled sample bottle on dissolved gas concentrations:

The introduction of a gas phase into a water-filled sample bottle changes the gaseous concentrations in the lake water. This is especially true in the procedure described below as the gas and liquid phases are shaken to equilibrium at the time of ^{14}C-methane injection. The effect on the concentration of gases in water by the introduction of a gas phase can be calculated as:

$$x = \frac{m}{1 + \alpha \frac{V_l}{V_g}}$$

where V_l is the volume of the liquid phase, V_g the volume of the gas phase, α the volume of the given gas (at STP) dissolved by one volume of

liquid at a given temperature and at 1-atm pressure, m the volume of the given gas (at STP) in the liquid and gas phases, and x the volume of given gas (at STP) present in the gas phase at equilibrium.

The volume of dissolved gas (at STP) in the liquid phase (y) at equilibrium is therefore given by:

$$y = m-x$$

Using the above equation the effect on in situ methane concentration at 4°C of the addition of 0.0006 ml of ^{14}C-methane in a 0.5-ml gas phase has been calculated. At an in situ methane concentration of 700 µM, the concentration would be reduced by 7.8%. At an in situ concentration of 1.0 µM it would be increased by 13.1%. It is believed that these concentration changes would not seriously affect methane oxidation rates in the presence of a substantial natural concentration of methane. However, at an in situ methane concentration of 0.1 µM the addition of this amount of label would increase the substrate concentration two times, thus oxidation rates calculated using in situ concentrations could be over-estimated. It has been our experience however, that oxidation rates at these low methane concentrations are usually very low or undetectable. Consequently in the consideration of lake budgets this error does not appear important. A similar calculation has been carried out to ascertain the effect on in situ concentration of the injection of a 0.5-ml gas phase containing 2% oxygen. At equilibrium the oxygen concentration in the sample water would be reduced by 8.5% at an in situ oxygen concentration of 7.0 mg/liter. At 0.5 mg/liter the oxygen concentration would be increased by 13.0%. Therefore, it is important that as small a gas phase as possible be introduced into the sample

bottles and that it be oxygen-free. A small gas phase minimizes the change in *in situ* gas concentrations and also permits a larger amount of added ^{14}C-methane be dissolved in the water phase at equilibrium, thus reducing the cost of the assay.

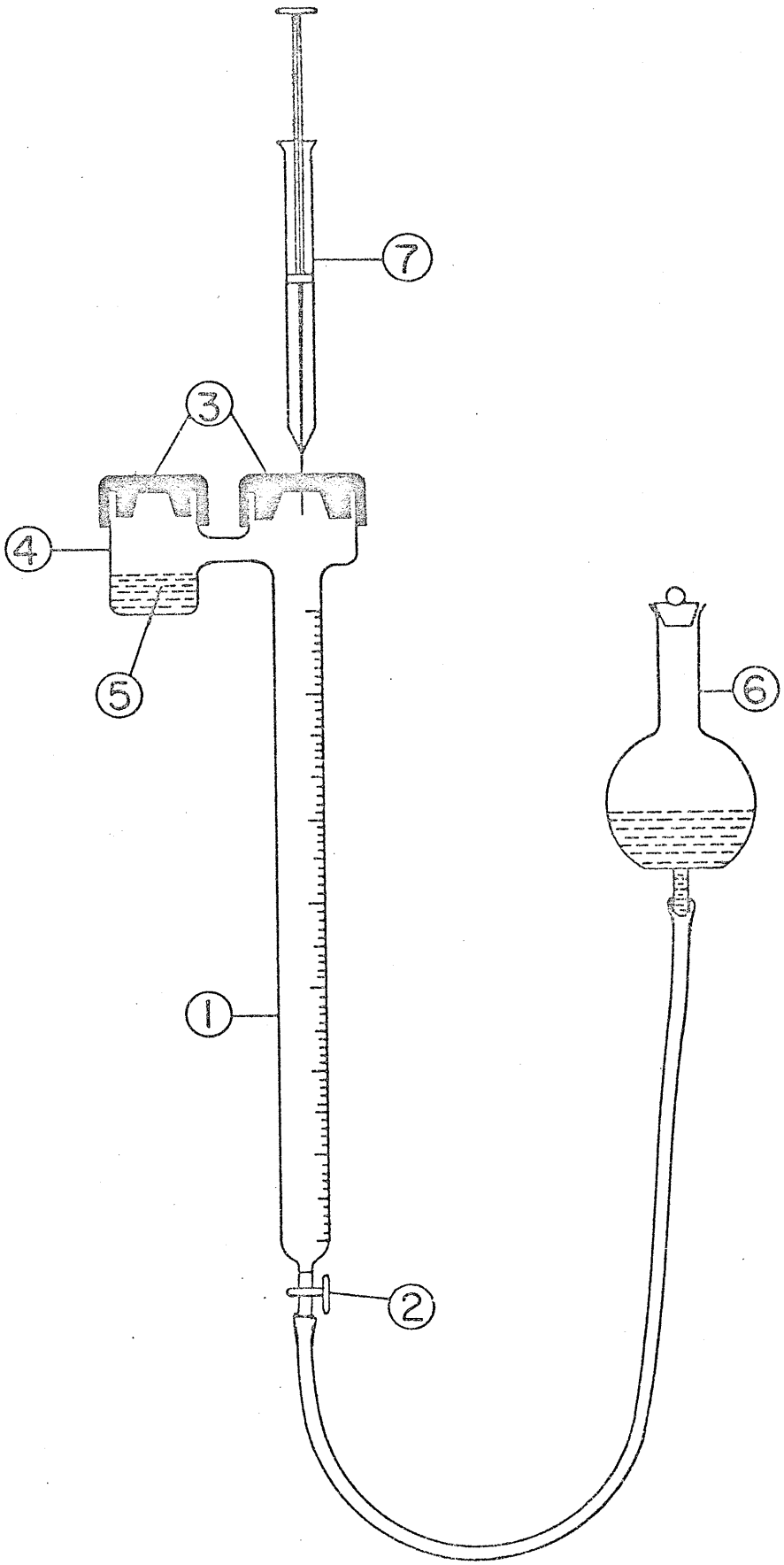
Preparation of the ^{14}C-methane label:

A gas mixture containing ^{14}C-methane (Amersham-Searle Corp.) was prepared in a mercury manometer (Fig. 1) as follows. With the mercury level set to zero, the dead volume was flushed with deoxygenated nitrogen (Macy *et al.* 1972). Alkaline-pyrogallol (Campbell & Evans, 1969) was added by syringe to the side arm of the manometer to maintain anaerobiosis. Atmospheric pressure was re-established by lowering the mercury level to compensate for the volume of the alkaline-pyrogallol. The required amount of ^{14}C-methane was then injected into the manometer with a one cc syringe which had been previously flushed with deoxygenated nitrogen (Macy *et al.* 1972). A volume of deoxygenated nitrogen was also added by syringe to obtain a suitable dilution of ^{14}C-methane activity. The mercury level was lowered again to compensate for the volume of added gases. Finally, 0.5 cc deoxygenated nitrogen was added to produce a slightly positive pressure, to preclude oxygen invasion. The gas mixture was allowed to stand overnight to ensure anaerobiosis.

Injection of ^{14}C-methane label:

As soon as the stopper had been removed from one of the pair of unfixed water samples taken from each depth, five cc was drawn into a syringe which had been previously flushed with deoxygenated nitrogen. This water was immediately added to the second sample thus filling the

Fig. 1. Apparatus used to dispense deoxygenated $^{14}CH_4$ constructed of: pyrex 10 ml pipette (1), fitted with a teflon stopcock (2); No. 25 "suba-seal" (3); the side arm (4) contained an alkaline-pyrogallol solution (5) to maintain anaerobiosis; a mercury reservoir (6) was constructed from a 100 ml volumetric flask with a ground glass stopper; the $^{14}CH_4$ nitrogen gas mixture was removed by inserting a 1 cc syringe (7) through the suba-seal.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neck of the second bottle and preventing air entrapment when the ground glass stopper was replaced after the addition of ^{14}C-methane.

One-half cc of the ^{14}C-methane gas mixture was drawn into a one cc syringe which had been previously flushed with deoxygenated nitrogen and the manometer was adjusted to maintain the gas pressure slightly above atmospheric. The second unfixed sample bottle was held rigidly at ∿30° angle and the labelled gas was introduced as a bubble trapped in the shoulder of the bottle. The bottle was held at that angle until the stopper had been replaced. This technique had been shown to permit anaerobic addition of ^{14}C-methane to water samples. Serum bottles were found to be unsatisfactory for this method because a small air bubble was often trapped under the serum stopper.

One-half milliliter of the ^{14}C-methane gas mixture was also added to the blank sample. It was then fixed immediately by increasing the pH to about 11.

The sample bottles and blank were shaken for 8 min. at maximum speed on a wrist shaker, which was sufficient to equilibrate the ^{14}C-methane label with the ^{12}C-methane present in the sample. The injection of ^{14}C-methane label and the shaking of serum bottles was carried out in diffuse light.

The samples and blank were incubated within 3°C of <u>in situ</u> temperature for from 1 to 5 hr, depending on the rate of methane oxidation. Incubation was in the dark to prevent photosynthetic oxygen production.

Analysis of incubated samples:

Following incubation the samples were fixed as before. The samples and blank bottles were opened and a 25-ml portion of each was analyzed for total methane concentration as described above. Three 10-ml portions

of each were also taken and processed as described below.

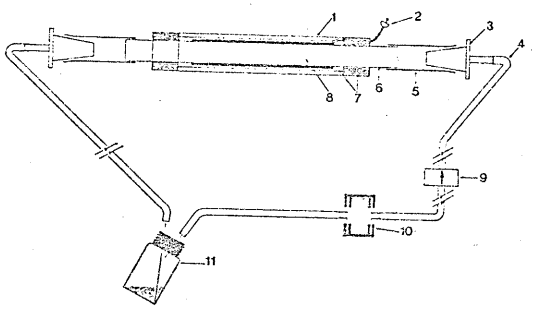
The first 10-ml portion was stripped of ^{14}C-methane by air scrubbing (~90 ml/min) for 5 min in the apparatus described by Schindler et al. (1972). No significant losses of ^{14}C-carbon dioxide were encountered (tested with added NaH ^{14}C O_3), presumably by reason of the high pH imposed. Four milliliters of this sample were then placed in 14 ml of a dioxane fluor (Schindler 1966).

The second 10-ml portion was similarly treated after adjustment to pH 2.5, low enough to ensure that all ^{14}C-carbon dioxide was lost. Both samples were counted in a scintillation counter and the appropriate blank value subtracted. As both carbon dioxide and methane were shown to be scrubbed from the second portion, the results from this sample were taken to represent the amount of methane carbon converted to particulate and soluble materials. The difference between the activity in the second sample and that in the first was taken to represent the amount of methane carbon converted to carbon dioxide.

The third 10-ml portion was used to establish the amount of ^{14}C-methane remaining after incubation. It was necessary to measure dissolved ^{14}C-methane concentrations for each sample since there was significant volumetric error during addition of ^{14}C-methane.

The 10-ml sample was drawn from the incubation bottles through a three-way valve into a 30-cc disposable syringe and stripped with 20 ml of helium as described earlier. The stripped ^{14}C-methane was then injected into a collection loop (Fig. 2), a modification of the apparatus described by Thompson and Hamilton (1974). This apparatus converted ^{14}C-methane to ^{14}C-carbon dioxide which was trapped in phenethylamine.

Fig. 2. Apparatus used to oxidize $^{14}CH_4$ and to trap the resulting $^{14}CO_2$ in phenethylamine. 1-15 mm-I.D. pyrex tube (16 cm long); 2-22-G Nichrome C winding to power source; 3-plastic reducer; 4-3mm-I.D. Tygon tubing; 5-6mm-I.D. silicone tubing; 6-6mm-I.D. quartz tube (20 cm long); 7-asbestos tape; 8-copper oxide; 9-sealed push pull air-pump; 10- glass injection port with serum stoppers; 11-scintillation vial containing 3 ml of phenethylamine and fitted with a silicone rubber stopper, pierced with 22-G 2.5 and 25-G 7.6 cm needles.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The injection procedure was as follows. The male opening of the three-way plastic valve on a 30-cc sample syringe (Fig. 3) was inserted into the female connector of a drying tube. A 5-cc plastic syringe (set at 4.0) was fixed to the third outlet of the three-way valve. With the three-way valve in position shown in Fig. 3 (closed to the atmosphere) the 26-G needle on the end of the drying tube was inserted into the injection port. The valve was then closed to the 5-cc syringe and a partial vacuum created by the air pump drew the ^{14}C-methane sample into the loop. When all the gas had been withdrawn from the 30-cc syringe the valve was opened to the 5-cc syringe, permitting 4 ml of air to flush the sample through the drying tube and into the loop. Injection of dry gases prevented the formation of a white, phenethylamine-water precipitate in the apparatus which caused clogging and poor replication.

Rates of methane oxidation could then be calculated by the following equation:

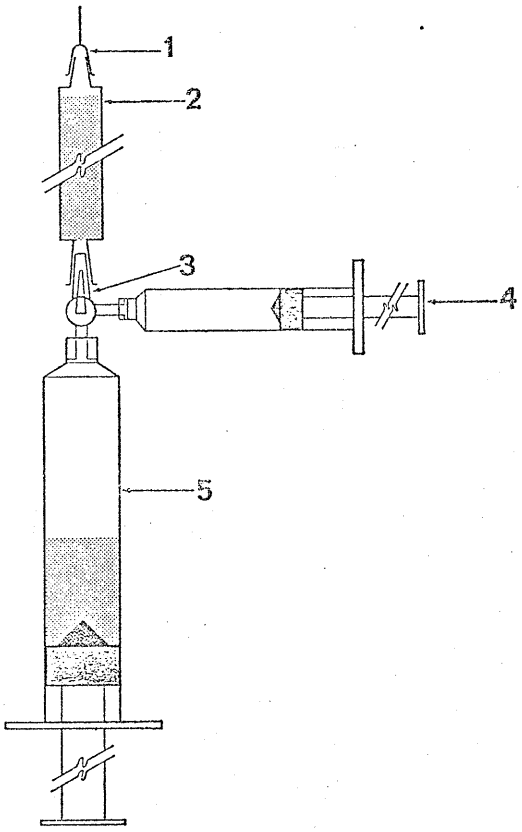
$$CH_4 \text{ oxidation } (\mu\text{Moles/L/hr}) = \frac{\frac{\text{Sample dpm/L}}{^{14}CH_4 \text{ dpm/L}} \times \underline{\text{in situ}}\ ^{12}CH_4\ (\mu\text{Moles/L})}{\text{hrs. of incubation}}$$

II A sampling method for obtaining methane concentration samples at precise and reproducible depths.

Introduction

The anoxic bottom waters of Lake 227 have a very steep methane gradient which is difficult to sample at precise and reproducible depth intervals over a long time period. The apparatus described below was successful in obtaining these samples.

Fig. 3. Apparatus used to inject dry gas sample into the collection apparatus. 1-26 G 1.3 cm needle; 2-magnesium perchlorate drying tube; 3-three-way valve; 4-5 cc plastic syringe holding 4 cc of air; 5-30 cc plastic syringe holding 10 cc of sample and 20 cc of helium.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Apparatus

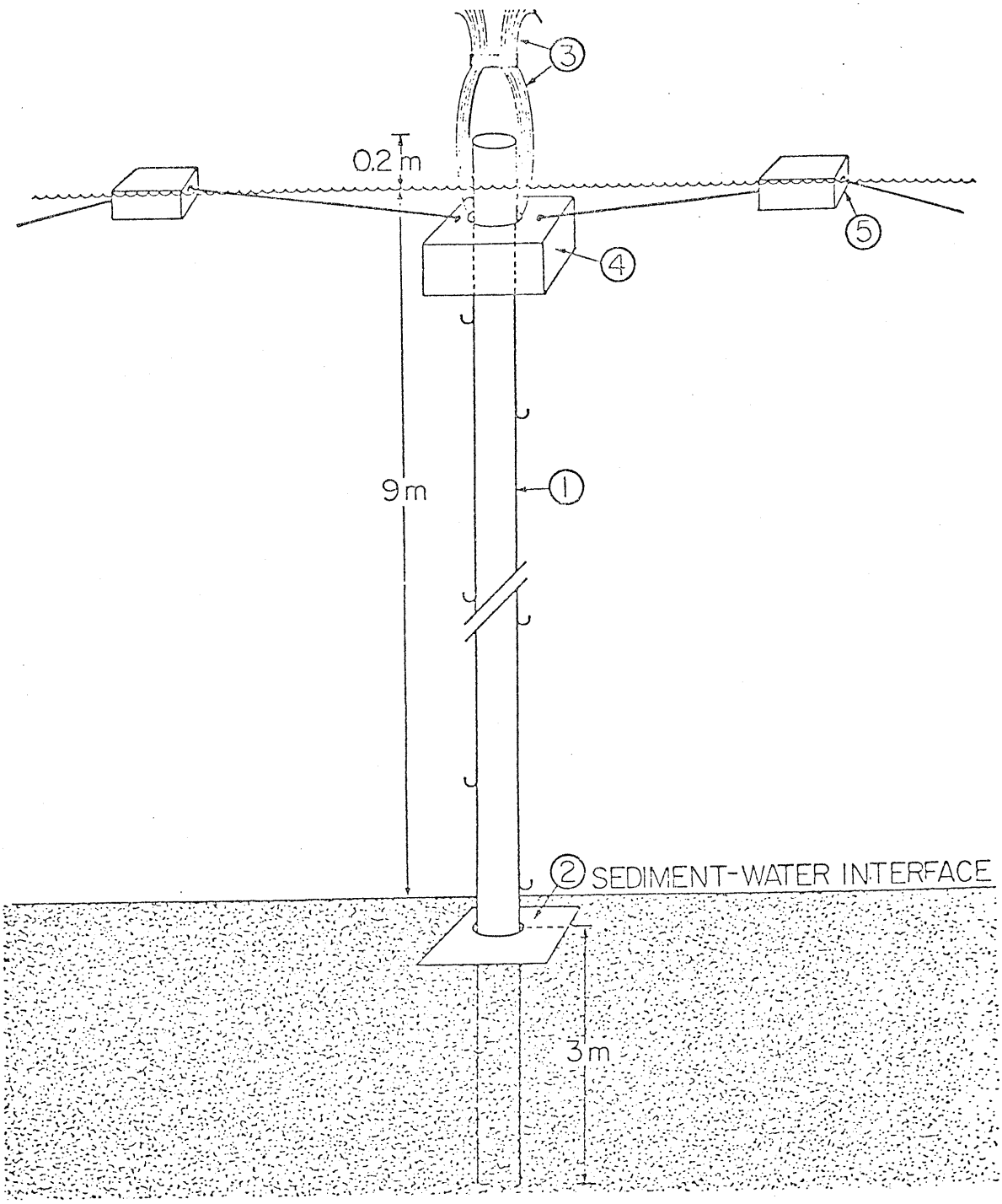
Polyethylene sampling tubes (Fig. 4) were supported by two lengths of light gauge Victaulic(R) steel water pipe, chosen because of its light weight (55 kg/7.6m) and superior ability to support its own weight with a minimum of bowing when set vertically in the lake. A plywood base at the sediment-water interface prevented the sampler from sinking too deep into the unconsolidated sediment. Separate lengths of polyethylene tubing for each sampling depth were fixed along the outside of the steel pipe, with openings at 1-m or 0.5-m intervals from the plywood base. A styrofoam collar provided sufficient upward tension on the pile to eliminate bowing, ensuring that the openings of the sample tubing were at the measured intervals. Three auxiliary surface floats at 120° intervals, attached to the styrofoam collar, provided rigid lateral stability without exerting a downward force on the apparatus.

Sampling and analytical procedures

Samples were collected by connecting a peristaltic pump to the desired sampling tubes by a short piece of Tygon tubing flushing, and pumping 25 cc directly into 50-cc glass syringes (known to be gastight for at least 12 hr). Air contamination by diffusion through the polyethylene sample tubing was not significant because of the short residence time (∿30 s) of the sample water in the tubing.

The samples were analyzed for *in situ* methane concentration as described in part one of this section.

Fig. 4. A vertical point sampler used to monitor dissolved methane concentrations in lake water (not to scale); 1-two lengths of 6.1 m x 6-cm OD x 5.9-cm ID Victaulic water pipe coupled by a 1-m x 8-cm OD length of standard gauge steel pipe and held in place by eight set-screws (not shown); 2-1-m^2 x 1-cm plywood base; 3-0.64 cm OD polyethylene sampling tubes; 4-central styrofoam float; 5-auxiliary styrofoam floats.


③
0.2 m
⑤
④
9m
①
② SEDIMENT-WATER INTERFACE
3m

Discussion

This sampler had several features which made possible the sampling of dissolved methane at precise and reproducible depths over long time intervals.

Sampling error was negligible; the 2% coefficient of variation of seven replicate samples from a single depth could be attributed to injection error of the gas chromatographic analysis. Because the sampler was calibrated from the sediment upward, samples could be obtained from precisely the same point of the water column on a long term basis, irrespective of lake level fluctuations. This is an important point because a sampling system which used the lake surface as a reference point would have introduced very significant errors since lake level fluctuations of 0.5 m were common.

Broenkow (1969) and Goering and Wallen (1967) have also used rigid supports to separate sampling distances. Our type of sampler was particularly useful in the highly stratified ELA lakes because it remained absolutely stationary in the lake for months and did not disturb the methane gradient during sampling. Thus relatively small increases in methane concentration could be precisely measured, making it possible to accurately monitor the accumulation of dissolved hypolimnetic methane during periods of stratification.

III Measurement of dissolved oxygen concentrations

Oxygen samples were obtained from a point source as described in part one of this section. They were pumped into 300-ml BOD bottles with multiple displacement to prevent atmospheric oxygen contamination. Samples were analyzed by the azide modification of the Winkler method (Am. Public Health Assoc. 1965).

RESULTS AND DISCUSSION

I Factors controlling rates of methane oxidation and the distribution of the methane oxidizers in a small stratified lake

Introduction

During periods of lake stratification methane oxidizing activity has been found at the oxic-anoxic interface of the water column (Rudd *et al.* 1974, and Patt *et al.* 1974). However, the physical and chemical factors which regulate the distribution and rates of activity of these bacteria on a yearly basis were unknown. Defining these parameters and establishing their importance is necessary to explain the contribution of methane cycling to whole lake metabolism. In this section five physical and chemical parameters (pH, temperature, oxygen concentration, methane concentration and lake stratification) are considered as possible controlling factors and ranked in order of their relative importance.

Methods

For some of the physiological experiments described in this section large samples were collected in two liter glass aspirator bottles. Multiple displacement was used to minimize invasion of atmospheric oxygen. These large samples were then partitioned into 125 ml reagent bottles with ground glass stoppers. Multiple displacement was again used to avoid contamination of the sample with atmospheric oxygen. Experiments with these sub-samples were always completed within five hours of sampling. Incubation was at *in situ* temperature unless otherwise stated while analyses of all sub-samples were carried out as

described in the general methods section. Measurement of in situ rates of methane oxidation were then carried out as described in the general methods section.

Results

Figure 5 shows methane oxidation rates in Lake 227 calculated on a whole lake basis (see part III of the results section). During summer stratification methane oxidation was observed only in a very narrow zone associated with abrupt changes in temperature as well as oxygen and methane concentrations (Fig. 6). Thus while oxidation rates at very specific depths could be quite considerable, activity during such periods did not contribute significantly to whole lake rates. In fact, 95% of the methane oxidation in Lake 227 occurred during and just after spring and fall circulation (Fig. 5) when methane oxidation occurred throughout the mixed portion of the water column.

Thus, in one instance oxidation was confined to a specific depth of the lake known to have sharp gradients in a number of important parameters and because of its narrow depth distribution the oxidation process was obviously under fairly rigid control. In the other case, during overturn, methane oxidation occurred throughout the water column yet under rather special circumstances (see later). It therefore appeared that on examination of the parameters known to have sharp gradients in the area of the thermocline, and which could also be followed during periods of overturn (temperature, pH, oxygen and methane concentrations) would enable us to determine the factors controlling rates of methane oxidation and the distribution of the methane oxidizing bacteria on a yearly basis.

Fig. 5. Whole lake rates of methane oxidation in Lake 227 during 1973 and 1974. Peaks of activity occurred during and just after spring and fall turnover.

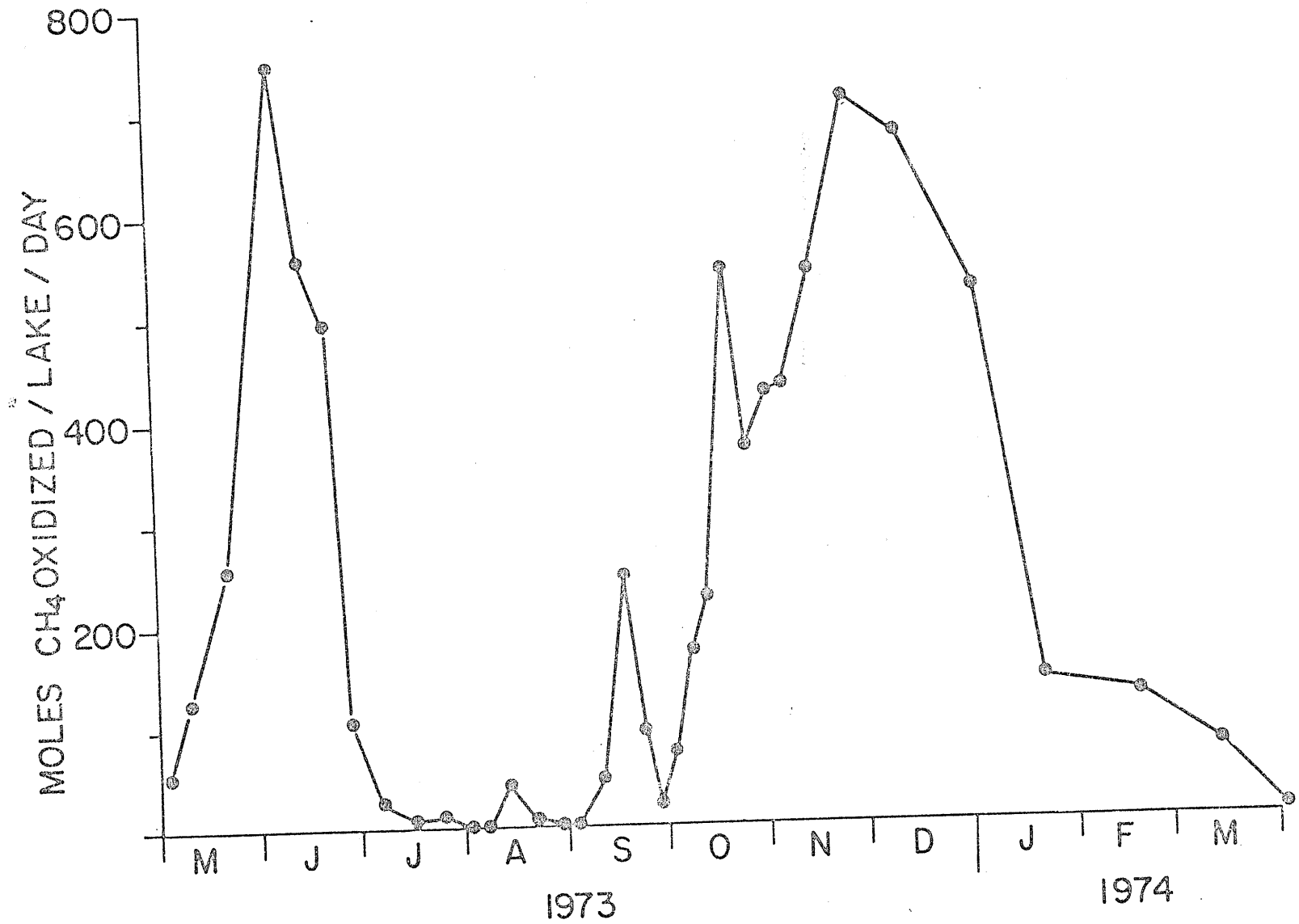
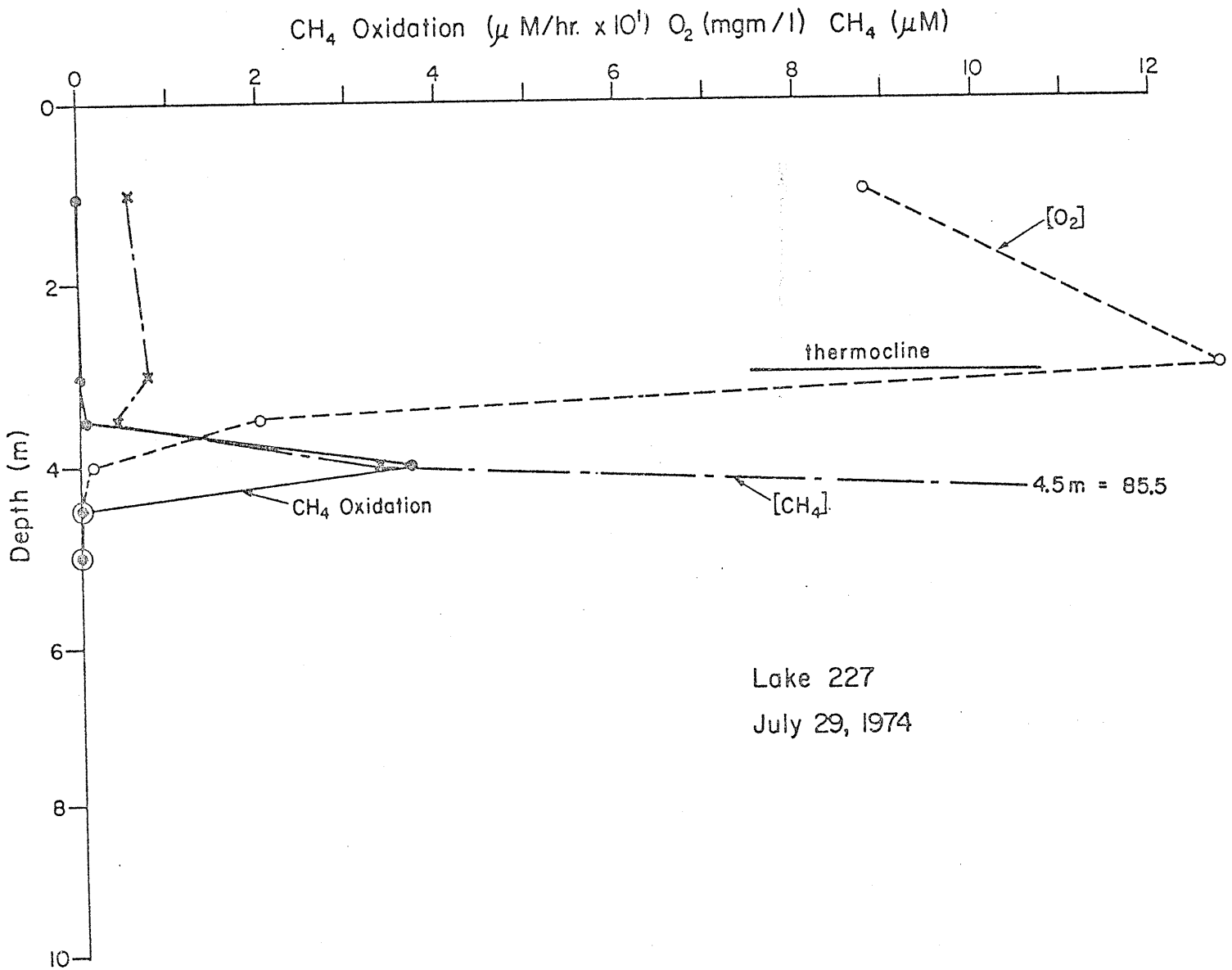

800
600
400
200
MOLES CH_4 OXIDIZED / LAKE / DAY
M
J
J
A
S
O
N
D
J
F
M
1973
1974

Fig. 6. A typical summer methane oxidation profile showing the lens of activity within the thermocline. The top of the thermocline is indicated by the solid labelled line.


CH4 Oxidation (μ M/hr. x 10¹) O2 (mgm/l) CH4 (μM)
0
2
4
6
8
10
12
Depth (m)
[O2]
thermocline
4.5 m = 85.5
[CH4]
CH4 Oxidation
Lake 227
July 29, 1974

Temperature:

As one would suspect in a biologically mediated process, temperature influenced rates of methane oxidation. During the few periods of time when field samples were collected at different temperatures which also had optimal concentrations of methane and oxygen (see later); we were able to observe a doubling of activity at specific depths within five days at 4°C, two days at 9°C and 1-2 days at 15°C.

In order to further resolve the effect a large sample was collected from the area of the thermocline in Lake 227 and sub-samples were assayed under various temperature regimes in the laboratory. As is shown in Fig. 7 methane oxidation rate increased with temperature from 2.0°C to about 25°C. Temperature also exerted an effect upon the partitioning of oxidized methane carbon between cell material and carbon dioxide. The percentage of methane converted to carbon dioxide increased linearly from 40 to 70% of the methane oxidized between 2°C and 33°C but dropped sharply at higher temperatures.

pH:

Another factor important in control of rates of biological processes and one which varied considerably with depth during periods of stratification was pH. To observe the effect of changing pH on the methane oxidizers a large sample at low oxygen concentration was collected from the thermocline of Lake 227 (in situ pH 6.3). The pH of sub-samples was adjusted with either deoxygenated sulphuric acid or sodium hydroxide solutions and rates of methane oxidation were assayed (Fig. 8). Methane oxidizing organisms appeared to tolerate acidic conditions but even slightly basic conditions significantly reduced oxidation rates.

Fig. 7. The effect of temperature changes on rates of total methane oxidation (closed circles), carbon dioxide production (open circles), and particulate production (crosses) in a sample from the thermocline of Lake 227 at 0.3 mg O_2/l

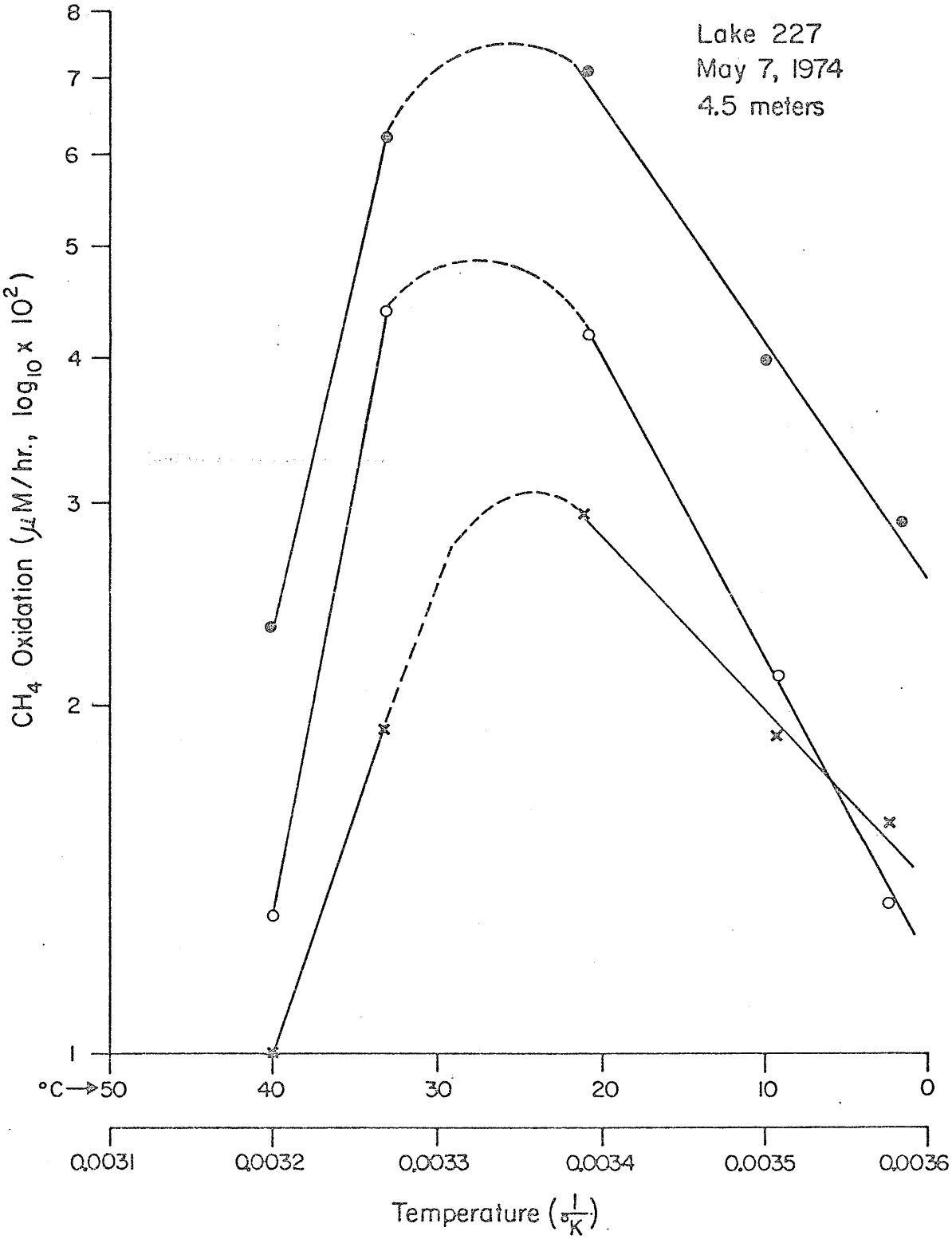
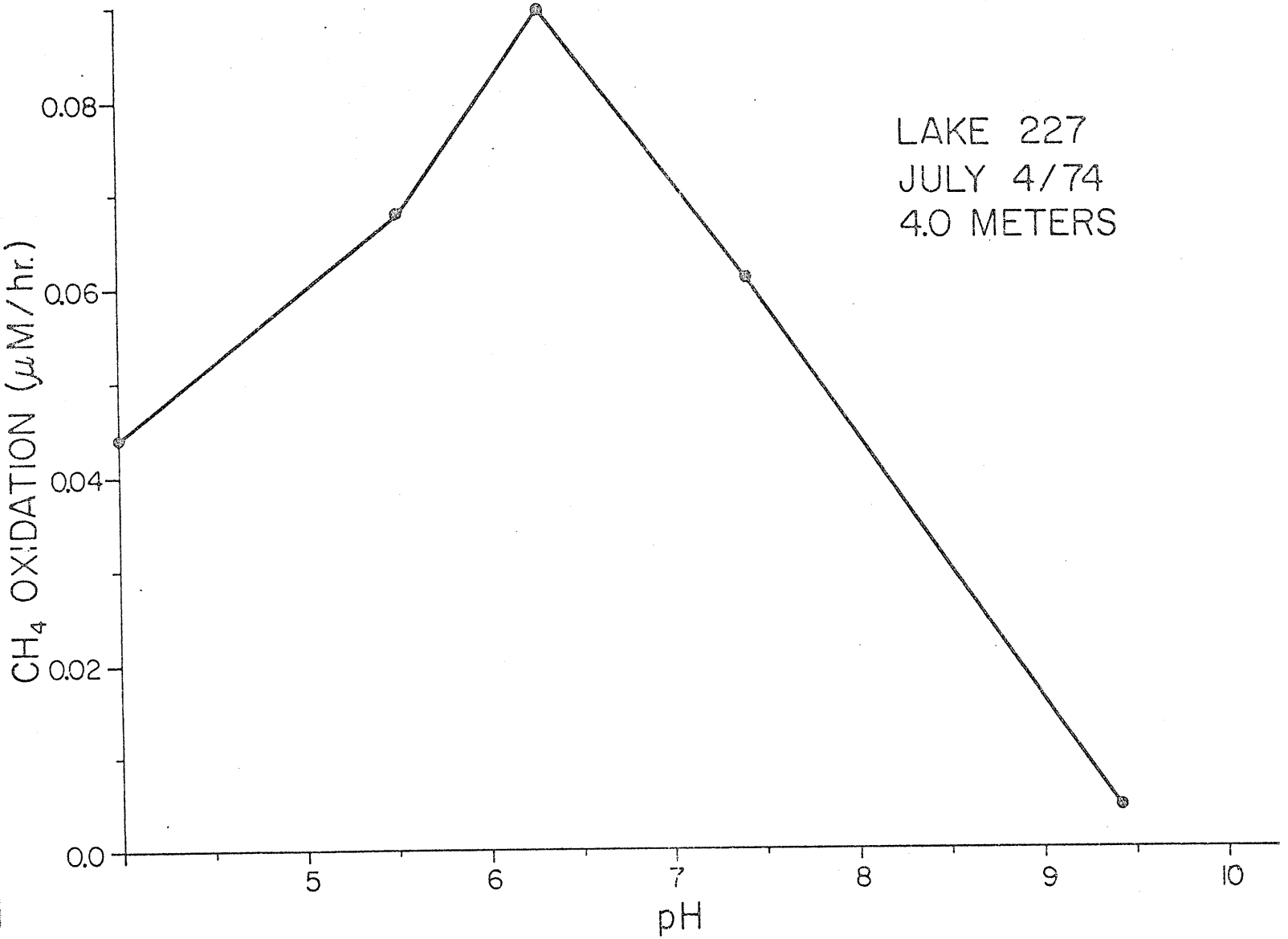

Lake 227
May 7, 1974
4.5 meters
CH_4 Oxidation (μM/hr., $\log_{10} \times 10^2$)
8
7
6
5
4
3
2
1
°C → 50
40
30
20
10
0
0.0031
0.0032
0.0033
0.0034
0.0035
0.0036
Temperature ($\frac{1}{K}$)

Fig. 8. The effect of pH change upon the rate of methane oxidation in a sample obtained from the thermocline of Lake 227.



Methane Concentration:

Concentration of substrate must also be suspected as a rate controlling factor in a microbially mediated process. As it was difficult to collect field data when all other conditions were optimal a large sample was again obtained from the depth of maximum activity in Lake 227 and determined the effect of various concentrations of methane on oxidation rates.

Methane concentrations in sub-samples were adjusted with de-oxygenated methane to within 0.37 - 38.3 μM, a range typical of observed zones of methane oxidation. The results are presented in Fig. 9 as a rate of reaction (μM/hr) versus substrate concentration (μM) (v vs. [S] plot). In order to determine the maximum rate (V_m) and the half saturation constant (K), the data was also plotted (Fig. 9) as v versus v/[S] after the recommendation of Dowd and Riggs (1965) that this method was superior to other methods of plotting, in that it exaggerates points which deviate from theory. In this case a small amount of methane (A) was present in the sample when collected. Therefore, the x intercept becomes Vm/K + A instead of Vm/K. From these data, Vm for this sample was determined to be 0.32 μM CH_4/hr under the experimental conditions and K + A was 4.8 μM CH_4. Since the in situ concentration of methane in this sample was 0.1 μM the K value was 4.7 μM.

From these data it can be deduced that methane concentrations below about 10 μM can significantly limit oxidation rates. A few of our field observations illustrate the effect. In Fig. 10a, for example, at a depth of 3.0 m the methane concentration was found to be 0.15 μM and the rate of oxidation was very low (0.0015 μM/hr).

Fig. 9. The inset presents the effect of increasing substrate concentration on the rate of methane oxidation in a water sample obtained from 4.25 m Lake 227 (6.0°C, 0.7 mg O_2/l) while the main figure presents a linear transformation of these data. The V vs [S] curve was fitted by using V_m + K and the equation $V = V_m[S]/K + [S]$.

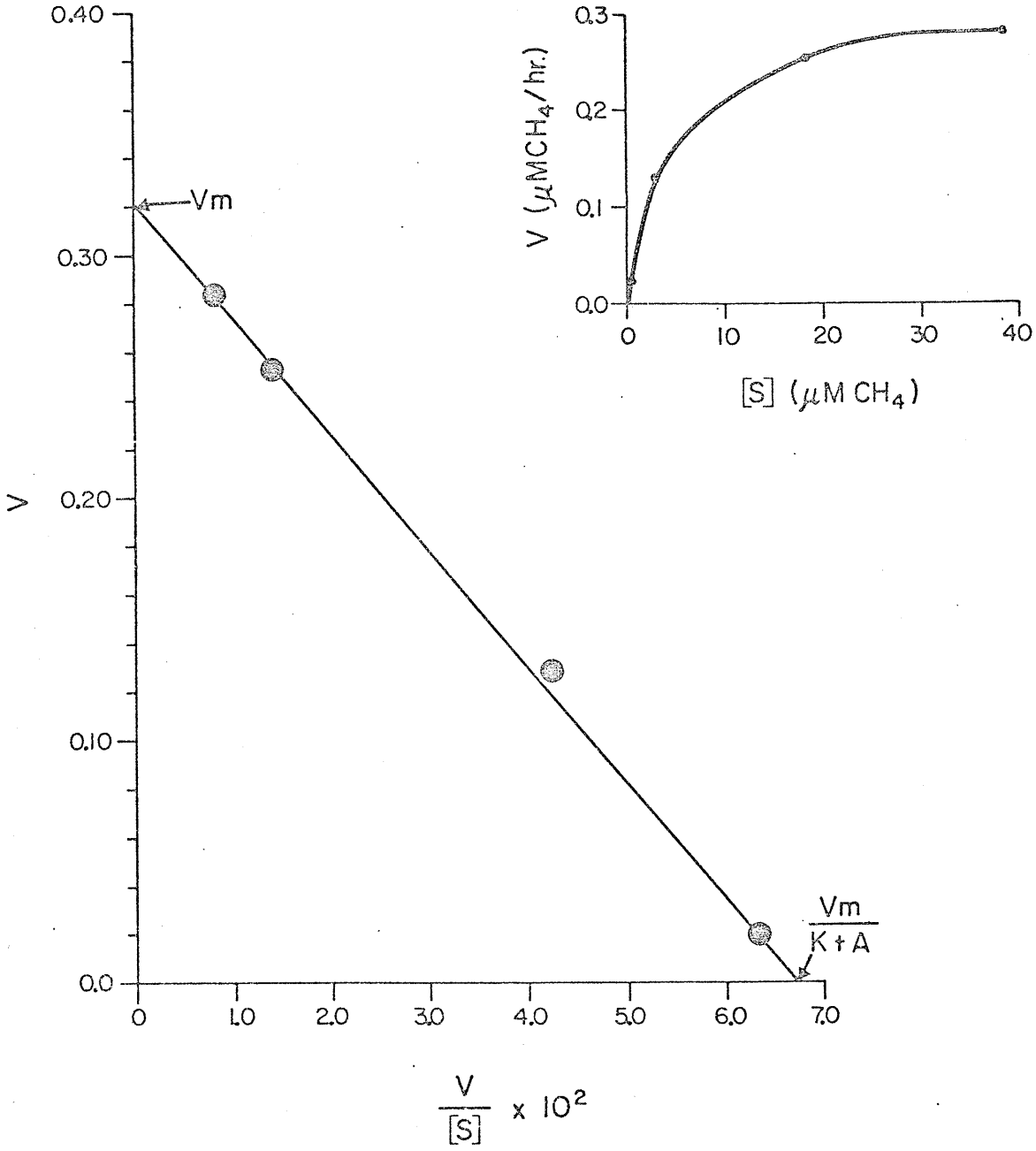
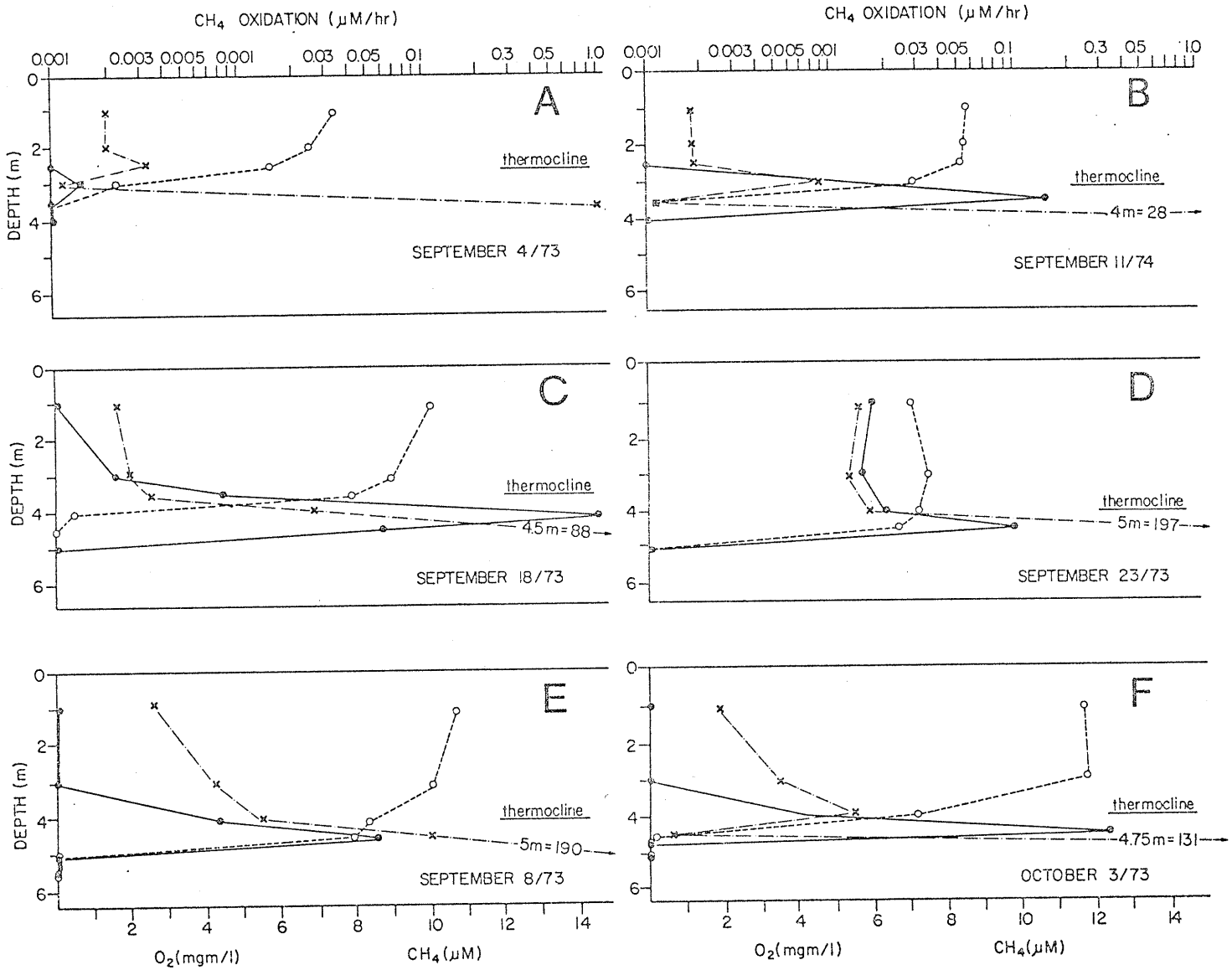

0.40
0.30
0.20
0.10
0.0
V
Vm
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Vm / K + A
V / [S] x 10²
0.3
0.2
0.1
0.0
V (μMCH₄/hr.)
0
10
20
30
40
[S] (μM CH₄)

Fig. 10. An example of the stimulatory effect of optimum oxygen concentration (0.1 - 1.0 mg/l) on rates of methane oxidation; rate of methane oxidation, closed circles; methane concentration, crosses; oxygen concentration, open circles. The top of the thermocline is indicated by a solid labelled line.


CH4 OXIDATION (μM/hr)
0.001
0.003
0.005
0.001
0.03
0.05
0.1
0.3
0.5
1.0
DEPTH (m)
A
thermocline
SEPTEMBER 4/73
B
thermocline
4m=28
SEPTEMBER 11/74
C
thermocline
4.5m=88
SEPTEMBER 18/73
D
thermocline
5m=197
SEPTEMBER 23/73
E
thermocline
5m=190
SEPTEMBER 8/73
F
thermocline
4.75m=131
OCTOBER 3/73
O2(mgm/l)
CH4(μM)

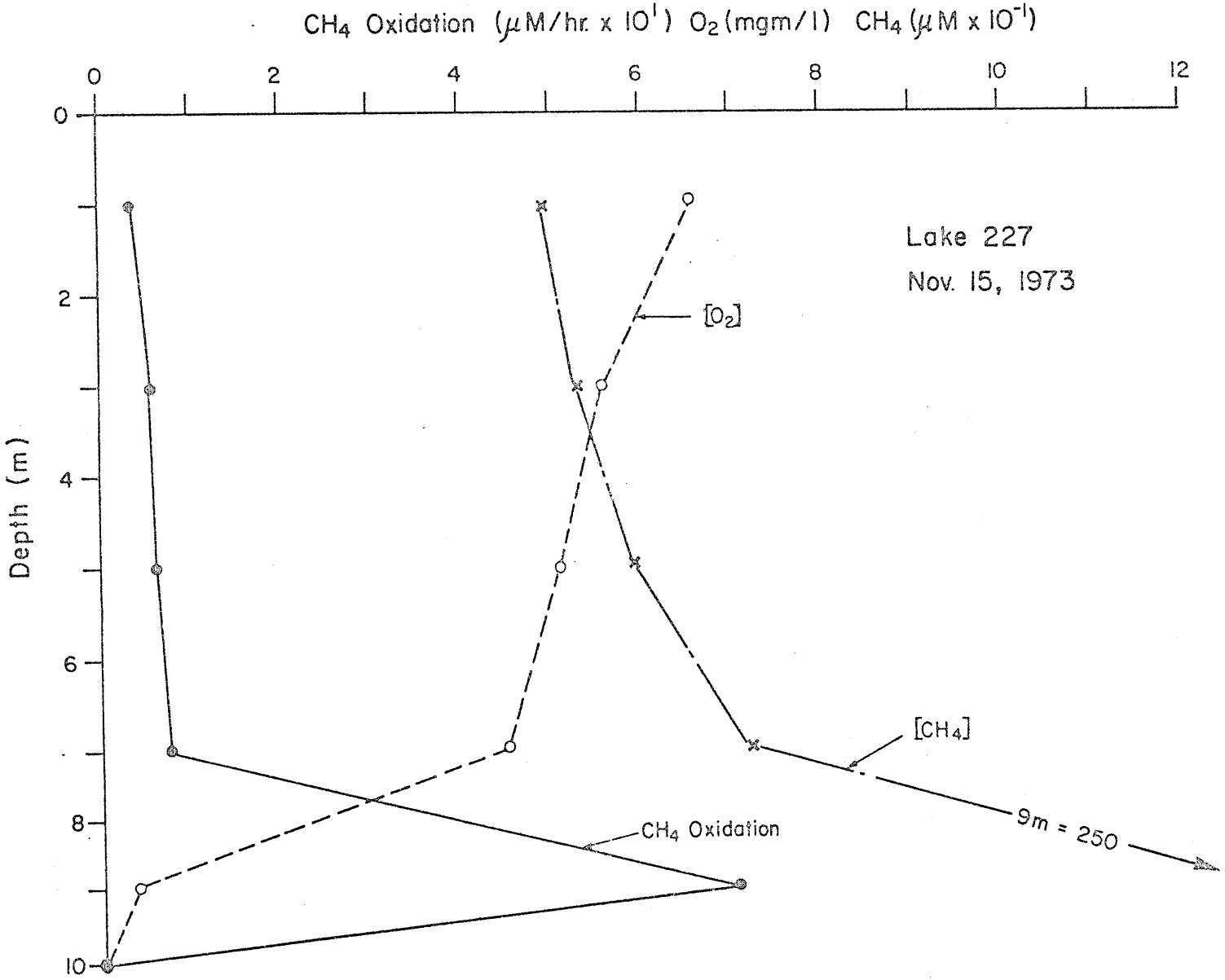
Similar cases of substrate limitation were seldom found in either the epilimnion or hypolimnion. In our experience it normally only occurred in the areas where oxygen levels were relatively low.

Oxygen Concentration:

As a result of these rather occasional instances of limitation due to low substrate levels, temperature, or pH, data on oxygen concentrations were examined. Published methods (eg. Whittenbury et al; 1970 and Naguib, 1971) for the isolation of methane oxidizing bacteria employ oxygen rich atmospheres yet it appeared from our data that such conditions were detrimental to maximal oxidation rates.

For example, in Fig. 11 the rate observed at 9m, at an oxygen concentration of 0.4 mg/l, was ten times as fast as the rate of 7m (4.5 mg O_2/l) even though the methane concentrations were observed to be adequate at both depths. Moreover during the period between September 4/73 and September 28/73 we observed a pulse in methane oxidation rates (Fig. 5) which appeared to be an example of oxygen acting as a rate limiting factor. In Fig. 10 a series of closely spaced observations obtained during this interval are presented. Figure 10a shows the typical narrow lens of activity associated with strong thermal stratification. Following this observation air temperatures dropped and the top of the thermocline eroded to about 3m (Fig. 10b) with consequent mixing, the introduction of small amounts of oxygen into what had been an anoxic zone, and the concomitant introduction of small amounts of methane into the oxygenated layers. Maximal oxidation rates increased by two orders of magnitude and the previously constricted lens broadened such that low rates were detected up to a depth of one meter. By

Fig. 11. A methane oxidation profile during fall turnover.

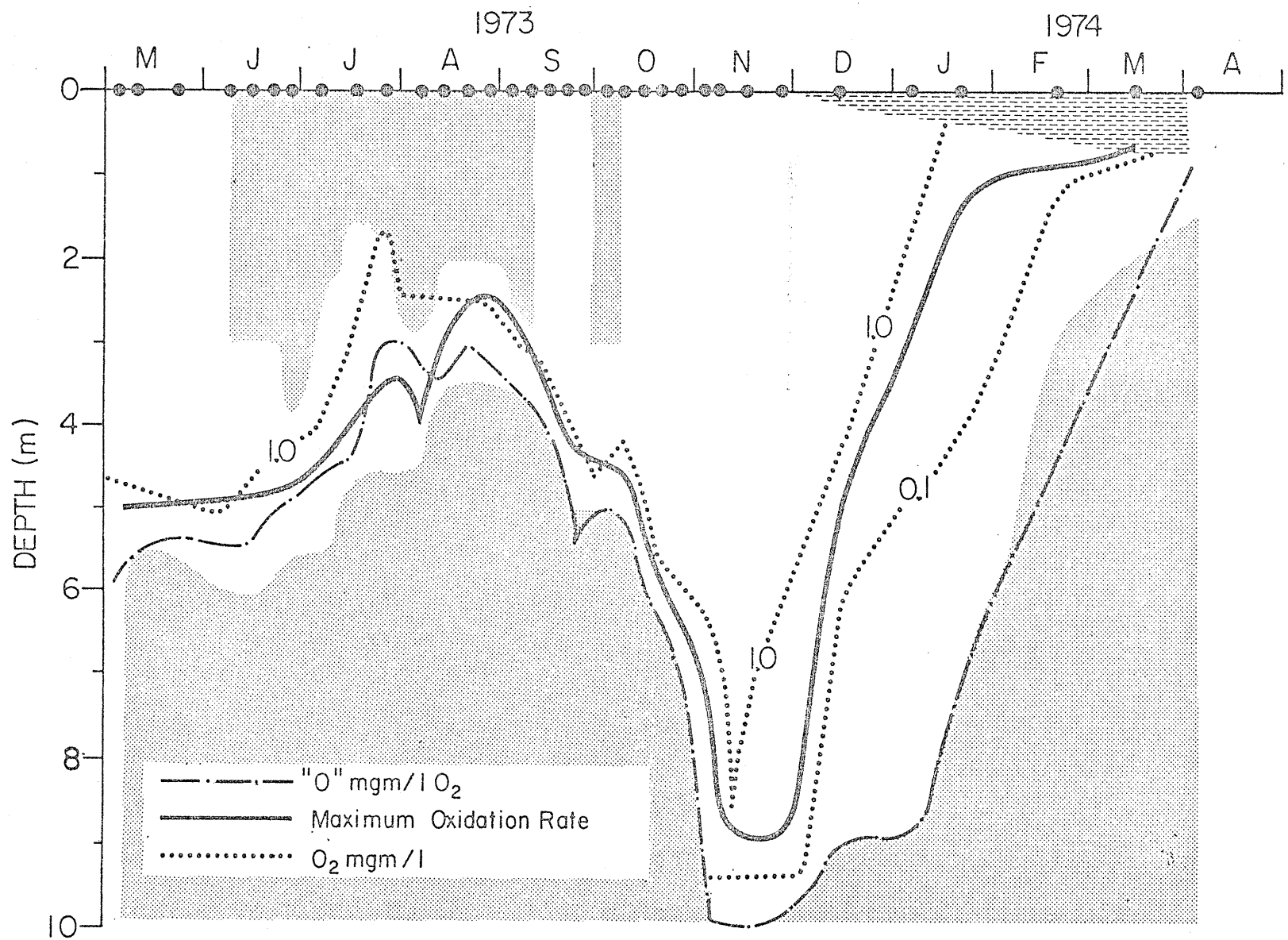

CH4 Oxidation (μM/hr. x 10¹) O2 (mgm/l) CH4 (μM x 10⁻¹)
0
2
4
6
8
10
12
Depth (m)
Lake 227
Nov. 15, 1973
[O2]
[CH4]
CH4 Oxidation
9m = 250

September 18 (Fig. 10c) this gentle mixing had depressed the thermocline to 3.5 m and the maximum rate had increased by a factor of seven, at an oxygen concentration of 0.5 mg O_2/1. It is important to note that this 700 fold increase in the maximum oxidation rate took place within two weeks, at low oxygen concentrations of between 0.08 and 0.5 mg O_2/1.

The mixing continued until September 23 (Fig. 10d), at which time the maximum oxidation rate occurred at 4.5 m (6.6 mg O_2/1). However, this rate was approximately eight times lower than previously observed maximal rate in spite of elevated methane concentrations. Mixing ceased by September 28 (Fig. 10e) and conditions stabilized such that a normal narrow lens of activity was re-established. However, maximal rates of oxidation continued to fall in the presence of relatively high levels of oxygen (7.9 mg O_2/1 at 4.5m) and the rates did not begin to increase until October 3 (Fig. 10f,5) when the oxygen concentration dropped to about 0.15 mg O_2/1.

As a result of such observations, the effect of oxygen concentration on maximum methane oxidation rate was examined on an annual basis (Fig. 12). The non-stippled area of Fig. 12 represents the zone of methane oxidation. It is evident from the zero oxygen isopleth that the lower limit of methane oxidation was associated with the depth of oxygen penetration. While maximum oxidation rates varied by over two orders of magnitude depending upon the time of year, it is evident that they closely followed the depth of zero oxygen concentration during spring, summer, fall and early winter. However, after November 25, 1973 the depth of zero oxygen and depth of maximum oxidation diverged. The reason for this was

Fig. 12. The stimulatory effect of optimum oxygen concentration shown on an annual basis; the zone of methane oxidation is shown as the non-stippled area.

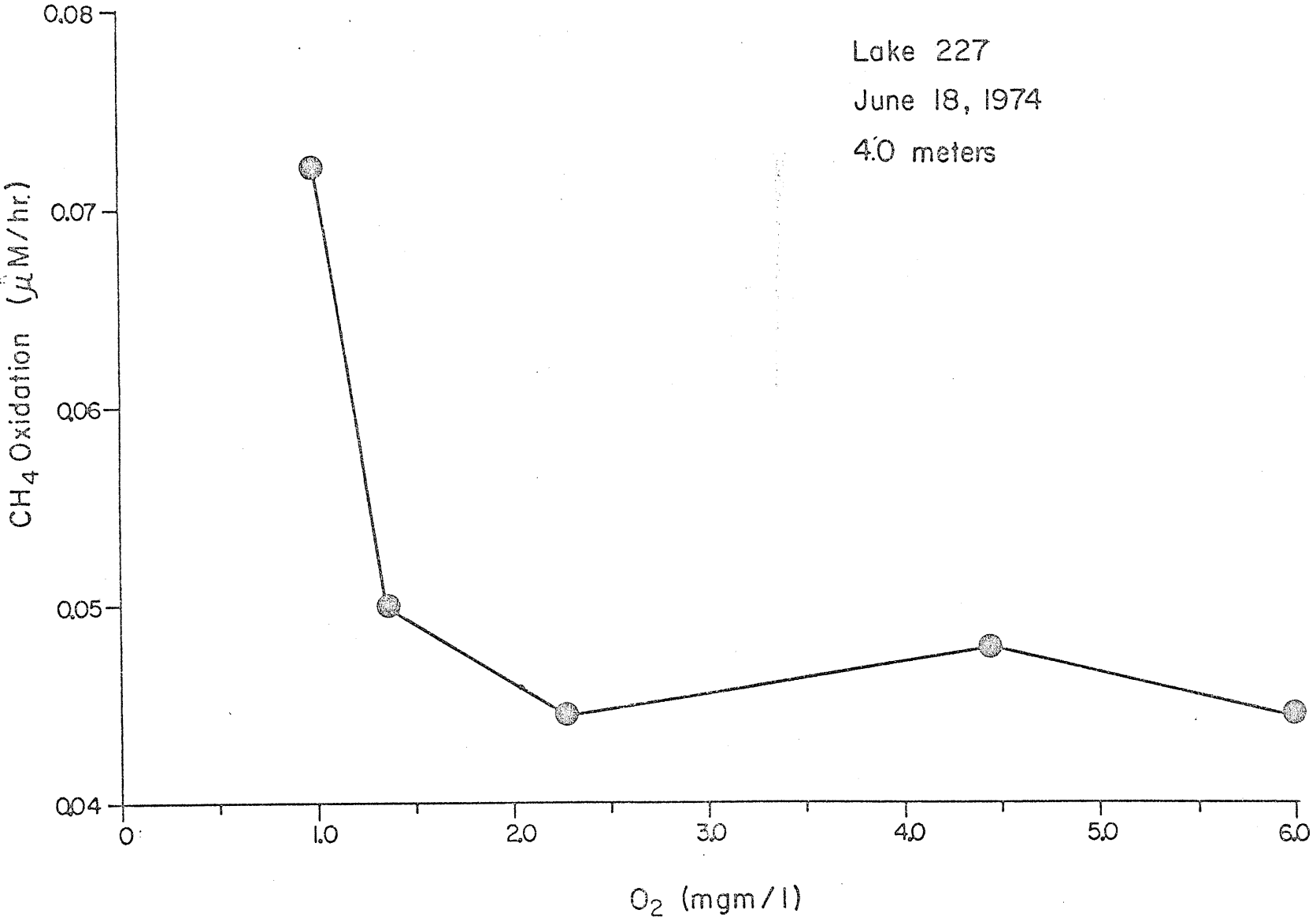

1973
1974
M
J
J
A
S
O
N
D
J
F
M
A
0
2
4
6
8
10
DEPTH (m)
1.0
1.0
1.0
0.1
"0" mgm/l O_2
Maximum Oxidation Rate
O_2 mgm/l

not immediately obvious until the 0.1 and 1.0 mg O_2/l isopleths were added. The methane oxidizers demonstrate an optimum in the range of 0.1 - 1.0 mg O_2/l, and throughout the whole of the year they operated maximally at oxygen concentrations of less than 1.0 mg/l.

These field observations were confirmed on a large sample (0.96 mg O_2/l) taken from the metalimnion (4.0m in Lake 227). When oxygen was added to sub-samples rates of methane oxidation decreased (Fig. 13). However, oxidation did not completely cease at the higher oxygen concentrations which is in agreement with field observations that oxidation continued for few weeks at vastly reduced rates in the epilimnion of Lake 227 after a spring partial spring overturn.

During periods of summer stratification we observed characteristic bimodel curves in the distribution of methane with depth (eg. Fig. 10a, b and f). High hypolimnetic concentrations (up to 2 mM) were due to the diffusion of methane from the bottom sediments (see part 3 of this section), coupled with conditions of complete anoxia. A very low minimum (frequently less than 0.1 μM) occurred in the vicinity of the thermocline due to the activity of the methane oxidizing bacteria and a second minimum was found near the surface of the lake. Between these two minima a second smaller maximum was observed in the epilimnion ($\sim$5 μM). Presumably, this latter maximum was due to horizontal diffusion of methane from the lake sediments in the epilimnion into waters containing elevated levels of oxygen where no methane oxidation was taking place. This characteristic methane profile has been used to quickly identify zones of methane oxidizing activity in other ELA lakes.

Fig. 13. The deleterious effect of increased oxygen concentration on rates of methane oxidation. The *in situ* oxygen concentration was 0.96 mg/l.


Lake 227
June 18, 1974
4.0 meters
0.08
0.07
0.06
0.05
0.04
CH_4 Oxidation (μM/hr.)
0
1.0
2.0
3.0
4.0
5.0
6.0
O_2 (mgm/l)

Discussion

The postulate that we observed psychrophilic populations of microorganisms is consistent both with the observation that these organisms were active at very low temperatures (0.1°C) and with the definition of psychrophiles offered by Ingraham (1962). That is to say, while the observed optimal temperature for methane oxidation was about 25°C (an apparently elevated optimum which has been noted for other psychrophilic bacteria by many other workers quoted in Ingraham, 1962) the slope of the Arrhenius plot shown in Fig. 7 is low and in agreement with the figures of Ingraham. Furthermore the "temperature characteristic" or "apparent activation energy" calculated from the Arrhenius plot is low. Our data yield a value of about 9,000 calories/mole; Ingraham also quoted 9,000 calories/mole for a typical psychrophile. It should be noted that the value for carbon dioxide production for this sample was about 12,000 calories/mole while the value for conversion of methane to cell material was about 7,000 calories/mole. This difference in the influence of temperature on conversion of methane to cell material and to carbon dioxide (Fig. 7) suggests that the two processes may be independently controlled.

From the temperature data in Fig. 7 there is no reason to suspect that methane oxidizers would not be active in the whole epilimnion of this lake during summer. However, such activity was not found and we conclude that temperature was not a primary control factor for the distribution of the methane oxidizers or for rates of methane oxidation. In our experience, temperature appeared to be a rate controlling factor only during a few periods of gentle mixing when oxygen and methane concentrations were optimal, (i.e. Fig. 5 September 11-18). Therefore

direct temperature effects can be discounted as an important control factor in whole lake considerations of rates of methane oxidation.

Similarly, control by pH can be discounted as a primary rate controlling factor since methane oxidation always occurred within the range of 6.0 - 7.1. Also, the distribution of methane oxidizers was not controlled by pH since values of approximately 7 occurred in the epilimnion for a period of six weeks during August and early September, 1973 (Fig. 12) and no methane oxidation occurred.

It is concluded that substrate limitation was unimportant in the control of annual lake methane oxidation since most methane was converted during periods of overturn, when the methane concentrations were greater than 10 μM. However, substrate limitation did occur in the lens of activity during periods of stratification but was only observed in conjunction with low oxygen levels. Thus the rate of oxidation within the lens and perhaps the shape of the lens may be determined by methane concentrations only if the oxygen concentration is optimal.

The apparent coupling of maximal rates of oxidation with oxygen concentrations between 0.1 - 1.0 mg O_2/l (Fig. 12 and 13) led us to the conclusion that these microorganisms are functional microaerophiles. However, this conclusion is not consistent with the fact that oxidation occurred throughout the oxygenated portion of the water column during periods of turnover (Fig. 11). One must then postulate either the presence of a different group of organisms during this period, a mechanism whereby the oxygen levels in proximity to the microbial cells were kept low or a capability of the population to continue methane oxidation at reduced rates under oxygen stress. While the development of a different flora during overturn cannot be discounted it seems unlikely in view of

the rapidity with which oxidation appeared in the upper waters during these periods. One of the last two alternatives or a combination of both is a more likely explanation.

During periods of fall overturn high concentrations of suspended material were often observed in the surface waters. Sediment transport into the water column during turnover, with subsequent redeposition has been observed in other lakes (Davis, 1973) while Emerson and Hesslein (1973) demonstrated movement of sediment in this lake. We therefore propose that the suspended sediment may provide microzones of low oxygen, analagous to those observed in soil particles (Greenwood & Goodman, 1967) which are suitable for the methane oxidizers. Indeed the presence of resuspended sediment in the surface waters proved to be an unerring indicator of methane oxidation in the upper water column. For example from September 10-25, 1973 during a period of rapid water circulation epilimnetic methane oxidation occurred (Fig. 12) and we observed high concentrations of resuspended sediment at the lake surface. Between September 25 and October 10 a warmer period resulted in re-stratification, an absence of resuspended sediment and cessation of epilimnetic methane oxidation. The situation was reversed once more after circulation recommenced on October 10.

While this effect may explain methane oxidation during fall overturn it should be noted that similar activity during and after spring overturn (Fig. 12) cannot be explained on this basis as no resuspended sediment was observed. Laboratory data (Fig. 13) indicated that methane oxidation rates are reduced in the presence of high oxygen and that these reduced rates can persist for long periods of time.

Therefore, it is reasonable to presume that both mechanisms are

responsible for methane oxidation in the surface waters during periods of circulation. While these rates are low compared to those observed in the associated lens (Fig. 11), it should be noted that when the volume development of the lake is considered this activity makes a very significant contribution to annual whole lake methane oxidation.

From a whole lake point of view the presence or absence of thermal stratification in Lake 227 was the ultimate factor controlling the rate of methane oxidation. During the summer the thermocline acted as an effective barrier reducing vertical diffusion of methane from and oxygen to the hypolimnion (Hesslein & Quay, 1973), thus reducing whole lake methane oxidation rates. By the end of the summer limnetic methane oxidation had virtually ceased as a result of the slow rates of vertical diffusion of dissolved gases within the thermocline. During spring and fall turnover mixing of anoxic water containing high concentrations of methane with oxygenated water as well as resuspended sediment vastly increased whole lake rates of methane oxidation (Fig. 5).

Attempts to culture these bacteria using the usual media and incubation conditions (Whittenbury et al. 1970; Leadbetter & Foster, 1958; Nauguib & Overbeck, 1970 and Cappenberg, 1972), were unsuccessful, as were attempts at isolation using a chemostat. The only positive culturing results were obtained when ^{14}C-methane oxidation was observed after sediment had been added to Whittenbury's NMS medium and Leadbetter and Foster's L medium. It is unlikely that the sediment contained any essential growth factors since the oxidizers also failed to grow on NMS or L media using lake water (obtained from an area of methane oxidation) as a base. Thus it was concluded that attempts to isolate the methane oxidizers were not

thwarted by toxicity of the media. Likely the high oxygen concentrations that we used (20% methane, 80% air) were inhibitory. We were however able to culture a "classical" methane oxidizer similar to Pseudomonas methanica (Dworkin & Foster, 1956) in a mud water enrichment culture that was incubated for several months. This suggests that the methane oxidizers that had been previously isolated by enrichment culturing techniques may not be the bacteria which are important as methane oxidizers in lake water and soils. The bacteria which dominate in our study lake appear to be adapted to low oxygen concentrations and so may never be isolated using the standard culturing techniques. A microaerophilic methane oxidizer would seem to have distinct advantages over aerobic species due to the steep gradients where methane and oxygen meet in nature, resulting in low oxygen concentrations in the presence of methane.

Summary

Several factors of varying importance influenced whole lake rates of methane oxidation in Lake 227. The presence or lack of thermal stratification was of ultimate importance since it controlled mixing of waters containing methane and oxygen. Ninety-five per cent of the whole lake methane oxidation occurred during spring and fall turnover (Fig. 5). Oxygen was another important control factor. During overturn high oxygen concentrations reduced oxidation rates in the upper part of the water column while rapid rates occurred at oxygen concentrations of less than 1.0 mg/l (Fig. 11). During summer stratification high oxygen concentrations prevented epilimnetic methane oxidation (Fig. 6,10,12,13) while anoxia prevented hypolimnetic oxidation (Fig. 6,10,12). Substrate concentration was a rate limiting factor at methane concentrations of less than

10 μM (Fig. 9). This only occurred in the narrow lens of activity present in the metalimnion during summer and only at optimal oxygen concentrations 0.1 - 1.0 mg/l (eg. Fig. 10a). Temperature was the primary control factor of methane oxidation rates on the few occasions when both oxygen and methane were within their optimum. The methane oxidizers active in this lake are characterized as being psychrophilic but with a temperature optimum of approximately 25°C (Fig. 7). Methane oxidation in Lake 227 occurred within a pH range of 6.0 - 7.1. Since these bacteria have a pH optimum of approximately 6.3 (Fig. 8) control of methane oxidation rates by pH was not observed.

II Factors controlling methane oxidation in shield lakes: the role of nitrogen fixation and oxygen concentration

Introduction

In part one of this section several factors controlling rates and distribution of methane oxidizers in Lake 227 were discussed. During summer stratification the microaerophilic methane oxidizers were inhibited by the high epilimnetic oxygen concentrations, thus methane oxidation was absent from the epilimnion despite adequate methane concentrations. Oxidation was confined to a narrow zone of activity within the thermocline where oxygen concentrations were low. A similar phenomenon in Lake Mendota has since been described by Patt et al. (1974).

An anomaly occurred during spring and fall overturn and throughout the winter which was inconsistent with the conclusion that the methane oxidizers were microaerophilic. During these periods methane oxidation continued relatively rapidly at high oxygen concentration throughout the

water column. Since it was believed that the same types of methane oxidizers were dominant in the lake throughout the year, two possible explanations for the anomaly were offered in the previous section. Dead particulate organic matter present in the water column at overturn and during the winter months, could have provided the microaerophilic oxidizers with microzones of low oxygen concentration. Alternatively, the oxidizers may have adapted to the high oxygen concentration and become oxygen insensitive under certain conditions. Evidence will be provided now that the latter case is correct.

Methods

Culture Procedures:

Lake water samples were obtained and transported to the lab as described in the General Methods section. The culture conditions were chosen to simulate as closely as possible the conditions which had been found to be optimal for methane oxidation in Lake 227 (part I of this section). Enrichment cultures composed of 50% lake water sample and 50% distilled water, plus 0.2 mM phosphate buffer (final pH 6.5 - 6.8) were continually bubbled with a methane (ultra high purity) air mixture at room temperature. The proportion of air in the influent gas mixture (~10%) was adjusted with a micrometering valve (Whitey) so that the oxygen concentration observed in the culture medium never exceeded 1.0 mg/liter as oxygen concentrations above 1.0 mg/liter have been found to be inhibitory to Lake 227 methane oxidation. The methane concentration in the cultures always exceeded 800 μM. The cultures were maintained by a weekly replacement of half the culture volume with fresh medium. These cultures were used as innocula for all subsequent

experimentation.

For some experiments the oxygen concentration of the culture medium was increased above 1.0 mg/liter. In this case additional pure oxygen was bubbled into the culture through a separate port in the culture vessel using a peristaltic pump for flow control. The cultures were stirred rapidly during these experiments.

Analyses:

Samples for all analyses were drawn from the culture flasks through glass sampling tubes directly into 50 cc all - glass syringes. These syringes were selected in order to prevent diffusion of atmospheric oxygen into the samples during incubation periods. Care was taken to flush the syringes and the sampling tube of all air bubbles before sampling.

Before determining the methane oxidation rates of the cultures the rate of oxygen consumption was determined by measuring the oxygen depletion with time in replicate sample syringes. Then the incubation time was adjusted (20-45 min) and enough room air was added to increase the initial oxygen concentration in the syringe to 1 mg/liter, ensuring that oxygen limitation did not occur in the syringes during the incubation period. Rates of methane oxidation were determined by a modification of the method described in the general methods section. Two-tenths ml of the ^{14}C-methane-nitrogen mixture ($\sim$100,000 dpm/ml) were added to a 35 ml sample in a 50 cc all glass syringe using a 1 cc syringe and 26 G x 2.5 cm needle. The syringe was shaken vigorously for five minutes to equilibrate the gas and the liquid phases. After incubation the sample was fixed by increasing the pH to 11 with NaOH.

The gas phase in the syringe was then expelled and the culture volume was reduced to 30 cc. Ten cc of this volume was transferred to a second 50 cc glass syringe through a female/female luerlock fitting. This portion of the sample was analysed for ^{14}C-methane activity and fixation of ^{14}C-methane as ^{14}C-carbon dioxide and ^{14}C-labelled cell material as described in the general methods section. The remaining 20 cc of sample was analyzed for methane concentration.

Nitrogen fixation was determined by measuring uptake of ^{15}N-nitrogen gas in syringes. Analysis of incorporated ^{15}N-nitrogen was carried out by emission spectrometry (Meyer et al. 1974) on a model NOI-5, N-15 Analyser (Statron). Samples were prepared by combusting directly in pure oxygen. The quantity of particulate nitrogen was determined as a function of pressure caused by the nitrogen gas produced (Flett, in preparation).

The dissolved oxygen concentration in the medium was determined on 20 ml syringe samples using a modification of Winkler method (Am. Public. Health Assoc., 1965). The accuracy was increased by titrating with a 1:10 dilution of phenylarsene oxide (Hach Chemical Co.). Replicate oxygen samples varied by a maximum of 0.05 mg O_2/liter. Ammonia, nitrate and nitrite were determined by the automated methods of Stainton et al. (1974). In situ methane oxidation profiles were obtained as described in the general methods section.

Results and Discussion

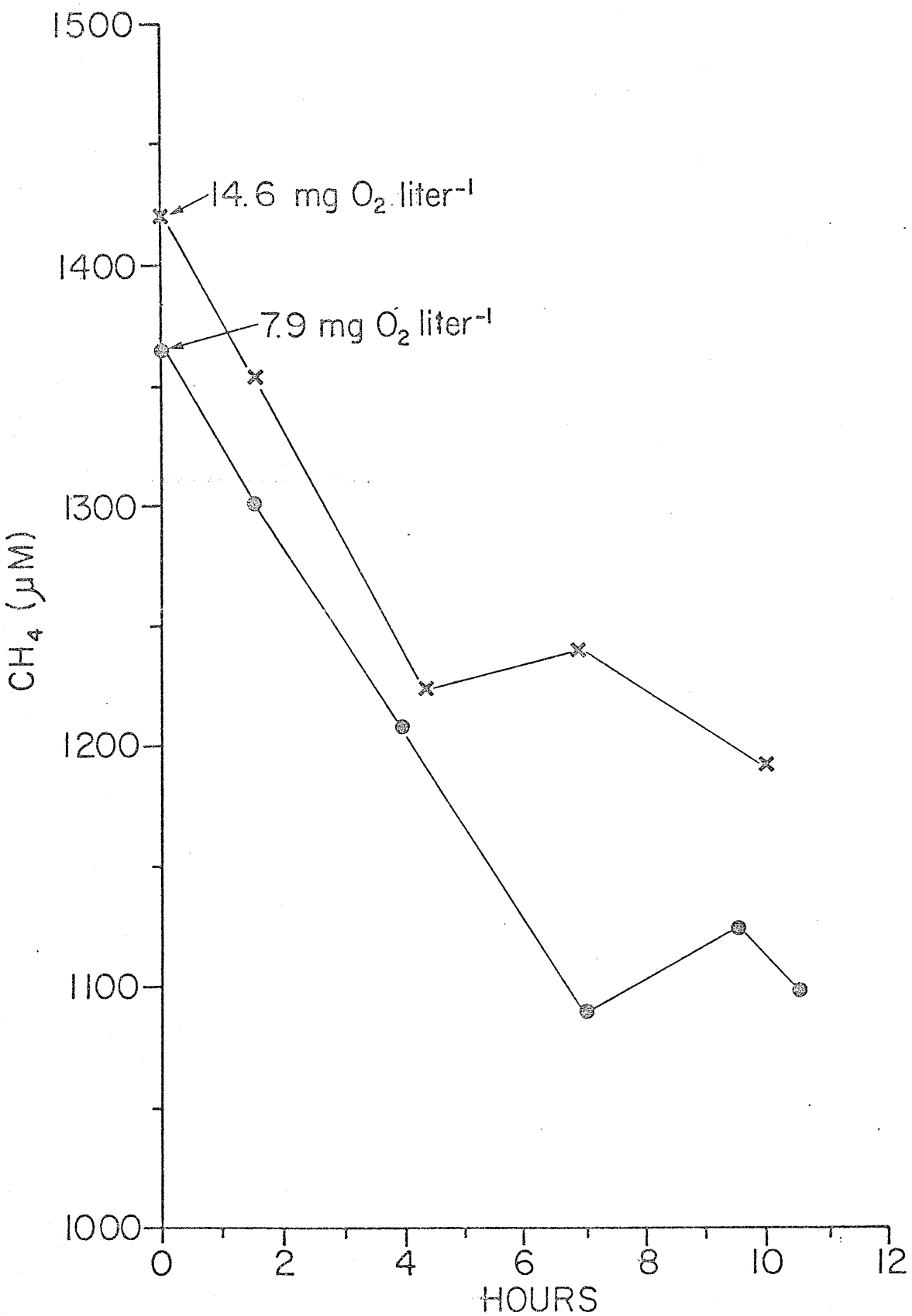
The methane oxidizing enrichment cultures require at least 5% Lake 227 water in the growth medium. The lake water contains a heat

sensitive organic compound(s) which loses its growth promoting capability during autoclaving or ultraviolet oxidation. Standard culture media (Whittenbury et al. 1970) and Leadbetter and Foster, 1958) do not support the growth of this culture. Additions of nutrient broth, yeast extract and soil extract to the standard culture media produce similar negative results probably due to the absence of the essential lake water factor(s).

In addition the oxygen concentrations in the 50% lake water medium must never exceed 1.0 mg/liter since higher oxygen concentrations inhibit methane oxidation both in culture and in lake water (part one of this section). The sensitivity of the methane oxidizing culture to oxygen concentration in excess of 1 mg/liter is demonstrated in Fig. 14. In this case methane oxidation was followed by determining the disappearance of dissolved methane with time in 25 cc replicate syringe samples obtained from the same enrichment culture. Sufficient oxygen was added to each of the syringes so that the initial oxygen concentration of the media was 14.6 mg/liter for one set of syringes and 7.9 mg/liter for a second set. Methane oxidation decreased abruptly in both cases after a few hours exposure to high oxygen concentration. The oxygen concentration at the conclusion of the experiment was in excess of 4 mg/liter in both cases.

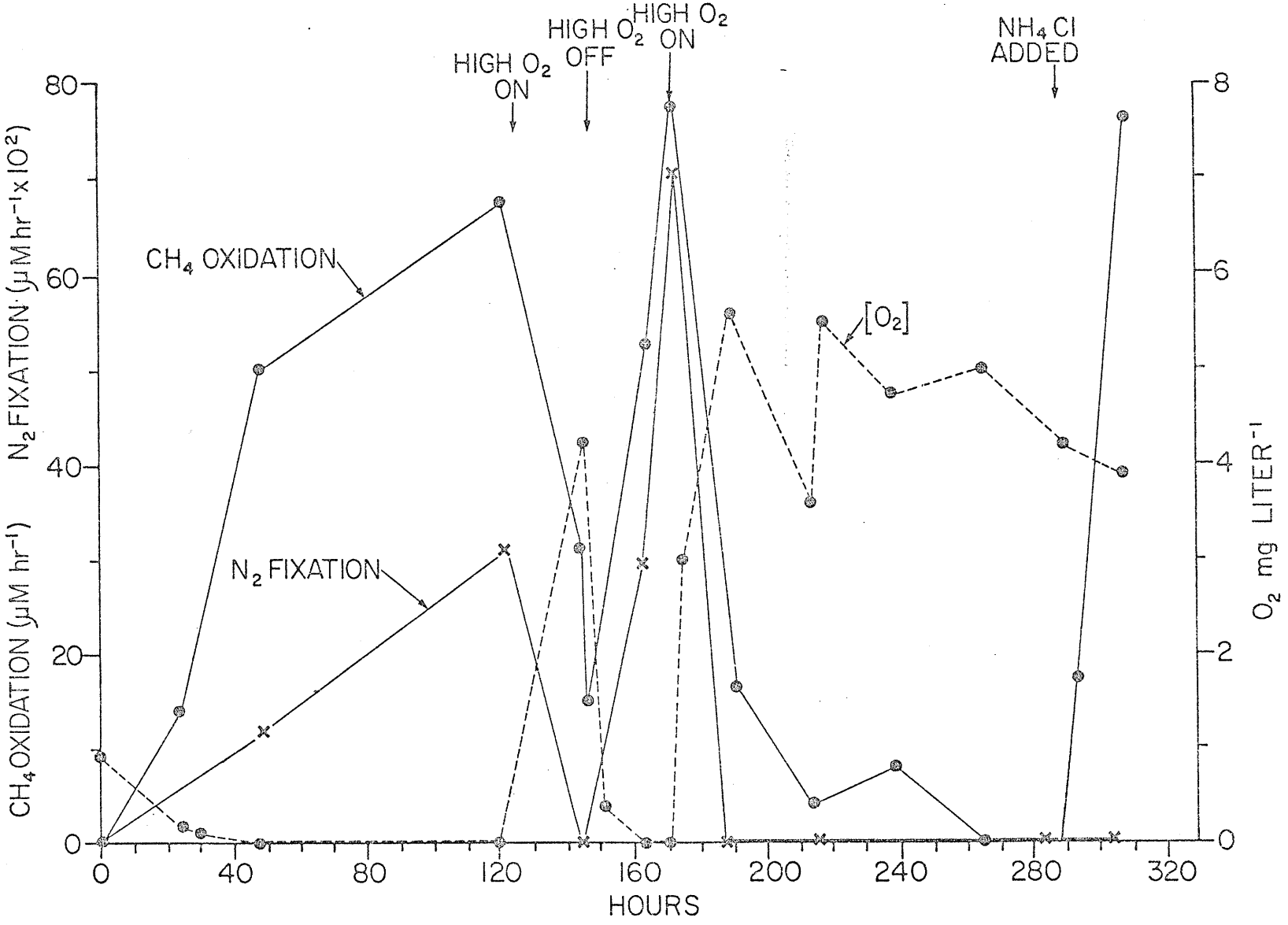
It is important to note that these oxygen concentrations were not immediately detrimental to the methane oxidizing culture (Fig. 14). This lack of immediate toxicity of high oxygen concentration suggested an indirect effect of high oxygen concentration on the methane oxidizing process.

Fig. 14. The inhibitory effect of high oxygen concentrations on a methane oxidizing culture grown on 50% lakewater medium.


1500
1400
1300
1200
1100
1000
CH_4 (μM)
14.6 mg O_2 liter^{-1}
7.9 mg O_2 liter^{-1}
0
2
4
6
8
10
12
HOURS

After ^{15}N analyses had shown that the culture was fixing significant quantities of dinitrogen the effect of high oxygen concentration on rates of both nitrogen fixation and methane oxidation were followed (Fig. 15) in a 50% lake water culture. After innoculation, methane oxidation (monitored by the $^{14}CH_4$ tracer technique) reduced the oxygen concentration in the medium to undetectable levels although oxygen was being bubbled into the medium continuously. The rates of methane oxidation and nitrogen fixation increased until 125 hours when the oxygen concentration was deliberately increased to above 1 mg/liter. By 144 hours at this high oxygen concentration nitrogen fixation had ceased, while the rate of methane oxidation had decreased more slowly (44% of the activity remained at 144 hours). The deleterious effect of high oxygen could be quickly reversed for both methane oxidation and nitrogen fixation by reducing the oxygen concentration in the medium to less than 1.0 mg/liter, (144 to 172 hours, Fig. 15). At 172 hours the oxygen concentration in the medium was increased again with the same results. In this case the high oxygen concentration resulted in a cessation of nitrogen fixation at 196 hours while methane oxidation did not completely cease until 264 hours. The absence of immediate lethal effect of high oxygen in this culture supports data presented in Fig. 14 and that obtained from in situ methane oxidizers (part I of this section). At 288 hours the dissolved inorganic nitrogen ($NH_4^+ + NO_3^- + NO_2^-$) concentration in the medium was very low (3 μM). This concentration was increased to 7 mM by the addition of NH_4Cl. There was an immediate rapid increase in the rate of methane oxidation in the presence of high oxygen concentration, indicating that the methane oxidizers had been nitrogen

Fig. 15. The effect of oxygen concentration on rates of methane oxidation and nitrogen fixation in an enrichment culture of methane oxidizing bacteria obtained from Lake 227. The culture was grown in 50% lake water medium.

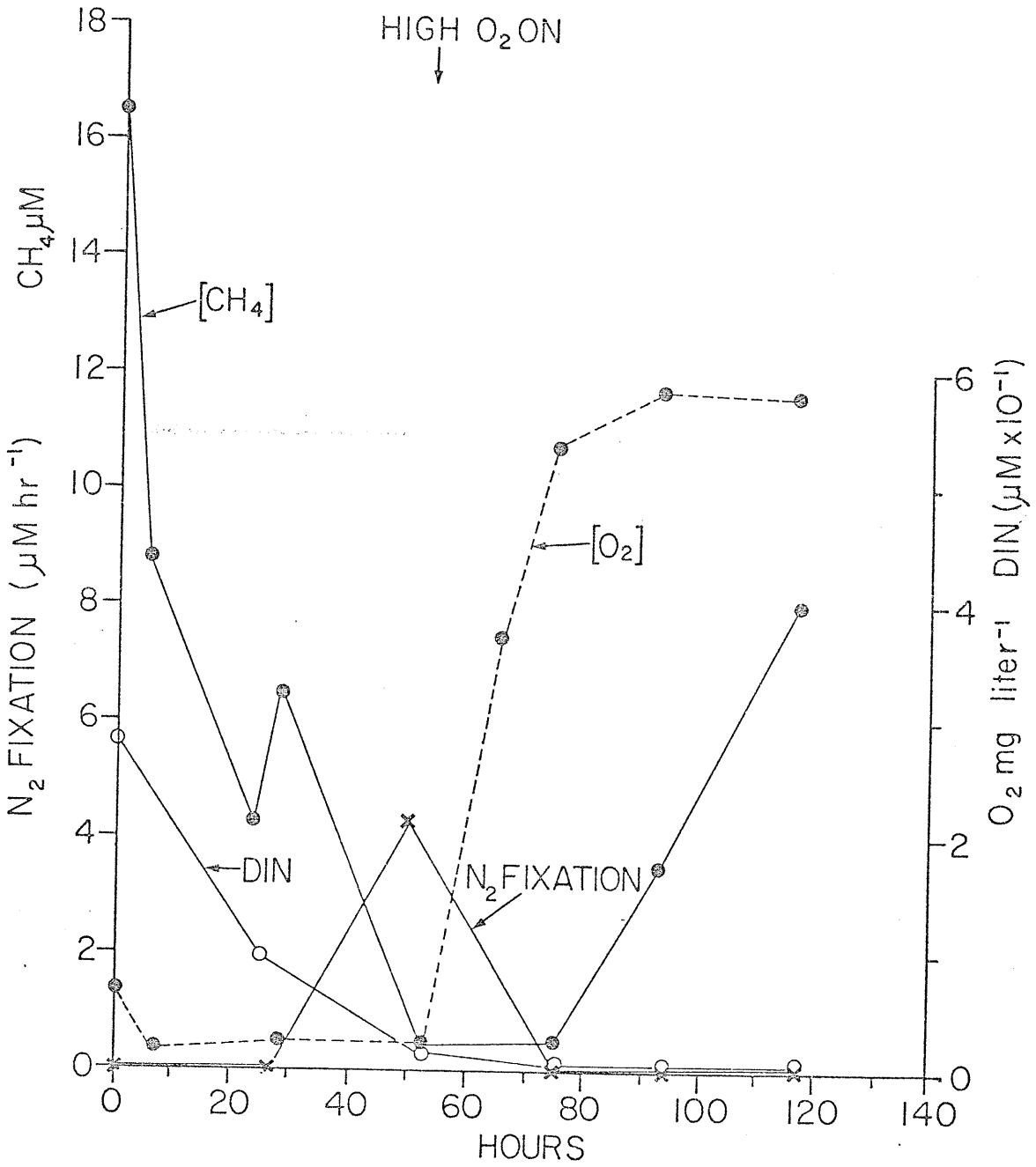

HIGH O_2 ON
HIGH O_2 OFF
HIGH O_2 ON
NH_4Cl ADDED
CH_4 OXIDATION
N_2 FIXATION
$[O_2]$
CH_4 OXIDATION ($\mu M\ hr^{-1}$)
N_2 FIXATION ($\mu M\ hr^{-1} \times 10^2$)
O_2 mg $LITER^{-1}$
HOURS
0
20
40
60
80
0
2
4
6
8
0
40
80
120
160
200
240
280
320

limited. Thus the oxygen sensitivity of the methane oxidizing process can be linked to the immediate toxic effect of high oxygen concentration upon the nitrogen fixation process. Direct evidence of this is the observation that the methane oxidizers are evidently not damaged by the high oxygen concentrations as they are very quickly reactivated by the addition of NH_4Cl. The persistence of methane oxidizing activity in the cultures (Fig. 14 and 15) and in lake water (part 1 of this section) for relatively long periods of time in the presence of high oxygen and low dissolved inorganic nitrogen (DIN) concentration may be explained by the presence of a residual dissolved or stored nitrogen supply which is eventually exhausted.

The addition of $NaNO_3$ to a nitrogen starved methane oxidizing culture at high oxygen concentration also resulted in an immediate rapid increase in the rate of methane oxidation. Thus nitrate will also replace nitrogen fixation as a nitrogen source enabling methane oxidation to continue at high oxygen concentrations. These observations have been confirmed by Whittenbury et al. (1975) who also found that ammonia rendered nitrogen fixing methane oxidizing cultures oxygen insensitive in continuous culture.

Methane oxidation in ELA lakes during summer stratification often occurs most rapidly at very low methane and oxygen concentrations (part 1 of this section). An attempt was made to simulate these conditions (Fig. 16) and to follow the effects of changes in oxygen concentration upon nitrogen fixation and methane oxidation at low methane concentrations. In this experiment the rates of methane oxidation were not measured by the ^{14}C-methane tracer technique since a significant amount of methane would have been consumed during the incubation period. Instead methane

Fig. 16. The effect of oxygen concentration on methane oxidation and nitrogen fixation in a 50% lake water culture receiving methane at a concentration equal to 30 μM in the medium.


HIGH O_2 ON
$CH_4 \mu M$
N_2 FIXATION ($\mu M\ hr^{-1}$)
O_2 mg liter^{-1} DIN ($\mu M \times 10^{-1}$)
[CH_4]
[O_2]
DIN
N_2 FIXATION
HOURS
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
120
140

concentration in the medium was used as an indicator of methane oxidation. The gas mixture (∿95 N_2, ∿3% CH_4, ∿2% O_2) bubbling through the medium maintained the methane concentration above 30 μM in the absence of methane oxidation. At 53 hours the methane oxidizing activity of the culture had reduced both the methane and dissolved inorganic nitrogen (DIN) concentrations to very low levels while rates of nitrogen fixation were increasing (Fig. 16). Increasing the oxygen concentration of the medium after 53 hours stopped nitrogen fixation and after a lag of 41 hours methane oxidation was also inhibited since the methane concentration of the medium began to increase. Thus at low methane and high oxygen concentrations methane oxidation was inhibited apparently because of a lack of available fixed nitrogen.

The methane oxidizing culture has several characteristics in common with those of the methane oxidation process in ELA lakes, (part 1 of this section). In both cases the methane oxidation rates were reduced after a few hours exposure to high oxygen at low DIN concentrations. However, they both remained active but at vastly reduced rates for relatively long periods of time in the presence of high oxygen and low DIN concentrations presumably under conditions of gradual nitrogen starvation. In both cases methane oxidation continued relatively rapidly at high oxygen concentrations when concentrations of DIN were also high. The methane oxidizers in both cases have also been found to metabolize ^{14}C-ethylene; this was reported for the lake water methane oxidizers in Flett et al. (1975) and we have found that the methane oxidizing culture metabolized 5.4% of added ^{14}C-ethylene within 20 minutes. Also, seemingly identical oxygen sensitive cultures have been obtained from

zones of oxidizing activity in Lake 227 during summer and winter. The summer samples taken at low in situ oxygen and DIN concentrations were oxygen sensitive while the winter samples had been actively oxidizing methane for months at high in situ oxygen and DIN concentrations. Enrichment cultures from both samples were dominated by a large "yeast like" microorganism in doublets and both summer and winter cultures were found to be sensitive to high oxygen concentrations at low DIN concentrations. For these reasons it is believed the methane oxidizing cultures were dominated by the same methane oxidizer which has been found to be responsible for the methane oxidation in Lake 227 throughout the year (part 1 of this section).

If this is true then the laboratory culture data can be used to explain factors controlling the rates and the distribution of methane oxidizing activity in ELA lakes during three very different periods of the year; summer stratification (Fig. 17), spring and fall turnover (Fig. 18) and under ice cover (Fig. 19).

The culture data presented in Fig. 16 corresponds to the conditions prevailing during summer stratification in Lake 227 (ie. presence of methane oxidation and nitrogen fixation at low DIN and oxygen concentrations and absence of oxidation and fixation when DIN is low and oxygen concentration is high). An example of a typical summer methane oxidation profile for Lake 227 is shown in Fig. 17. A very narrow zone of methane oxidizing activity occurred between 4.25 and 5 meters where the oxygen concentration was less than 1.0 mg/liter. There was no oxidation below 5 meters because of anoxia. Methane oxidation was absent above 4.25 meters because of the inhibitory effect of high oxygen concentrations

Fig. 17. A typical methane oxidation profile during summer stratification demonstrating the narrow zone of activity occurring at oxygen concentrations of less than 1 mg/liter.

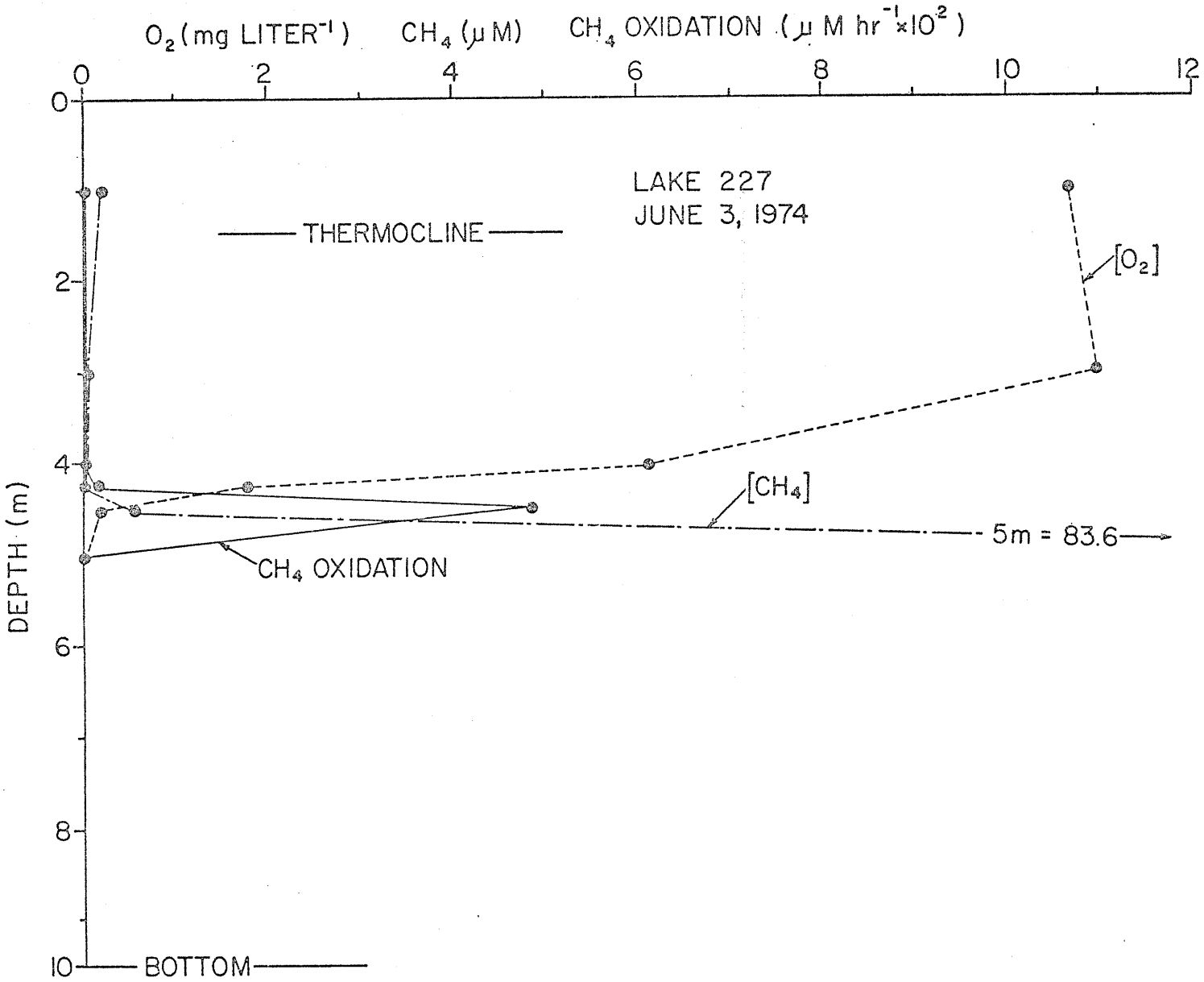

O_2 (mg LITER^{-1})
CH_4 (μM)
CH_4 OXIDATION (μM hr^{-1} $\times 10^{-2}$)
0
2
4
6
8
10
12
LAKE 227
JUNE 3, 1974
THERMOCLINE
[O_2]
[CH_4]
5m = 83.6
CH_4 OXIDATION
DEPTH (m)
BOTTOM

Fig. 18. A typical methane oxidation profile taken just after fall turnover showing a rapid rate of methane oxidation throughout the water column in the presence of high oxygen concentration.

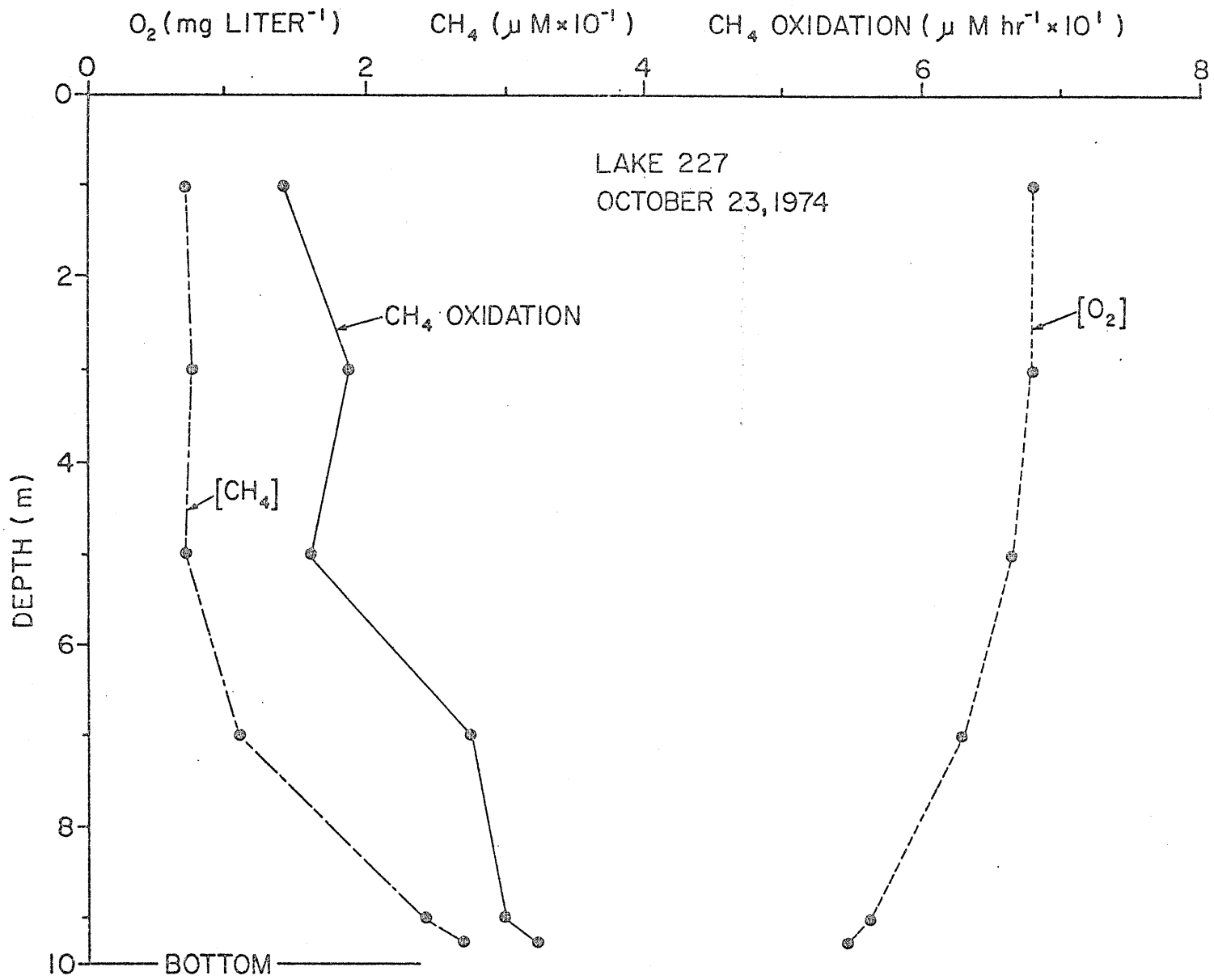
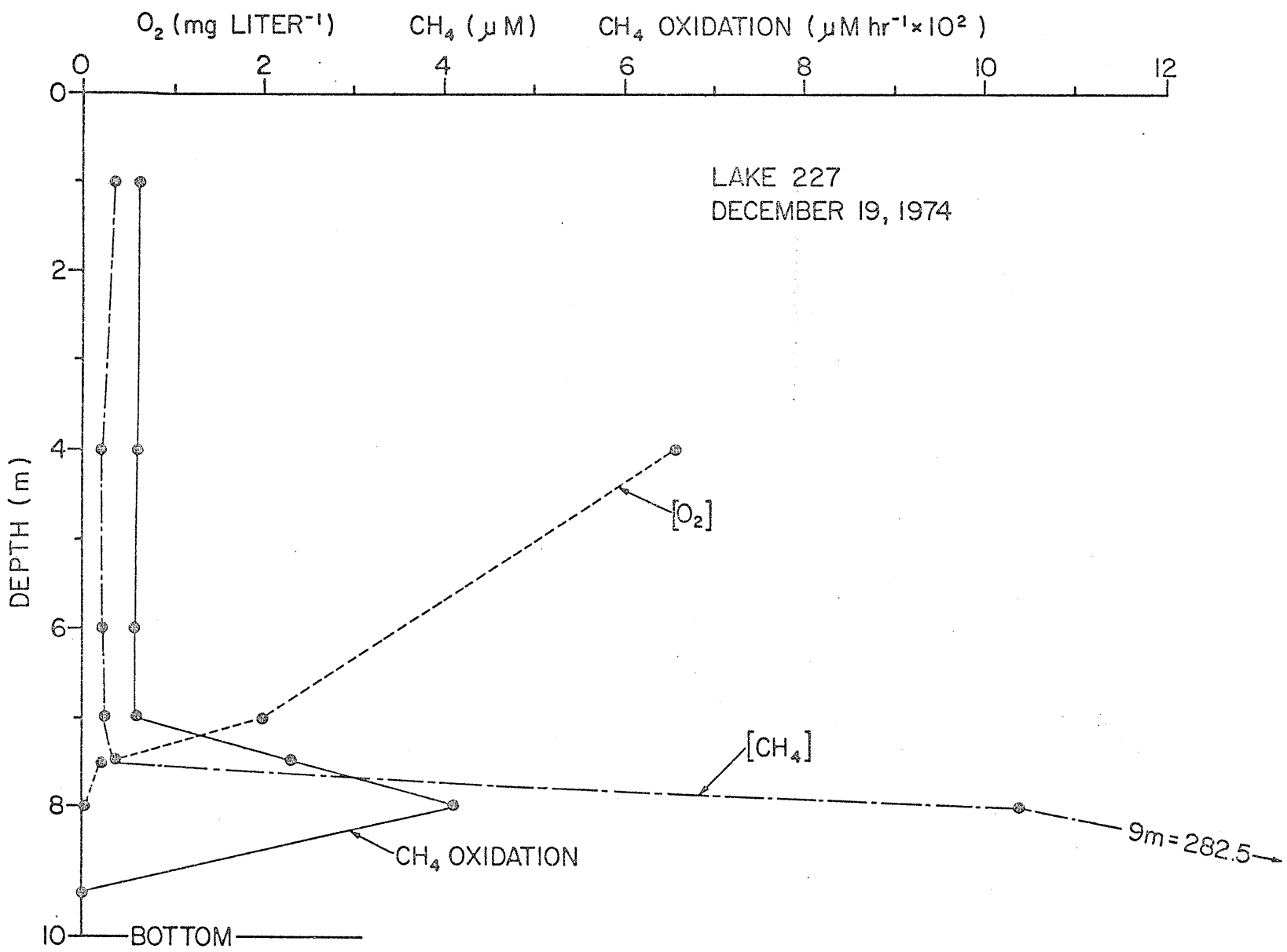

O_2 (mg LITER^{-1})
CH_4 (μ M $\times 10^{-1}$)
CH_4 OXIDATION (μ M hr$^{-1} \times 10^{1}$)
0
2
4
6
8
LAKE 227
OCTOBER 23, 1974
CH_4 OXIDATION
$[O_2]$
$[CH_4]$
DEPTH (m)
0
2
4
6
8
10
BOTTOM

Fig. 19. A typical winter methane oxidation profile showing continuing methane oxidation under ice-cover in the presence of high oxygen and DIN concentrations.


O_2 (mg LITER^{-1})
CH_4 (μM)
CH_4 OXIDATION (μM hr^{-1} × 10^2)
0
2
4
6
8
10
12
LAKE 227
DECEMBER 19, 1974
DEPTH (m)
[O_2]
[CH_4]
9m = 282.5
CH_4 OXIDATION
BOTTOM

as explained in part 1 of this section. A more refined explanation of the factors controlling the distribution of the methane oxidizers in this lake (and all other ELA lakes investigated) can now be given. The absence of epilimnetic oxidation was not due to a lack of methane since epilimnetic methane concentrations gradually increased to ∿5 μM towards the end of summer stratification (part 1 of this section). Instead, the absence of epilimnetic methane oxidation in Lake 227 during summer stratification can be explained by the very low epilimnetic DIN concentrations (∿2 μM) (Schindler et al. 1974). Since the alternate nitrogen source, nitrogen fixation, was not functional at high epilimnetic oxygen concentrations the methane oxidizers in the epilimnion were gradually inactivated as a result of nitrogen starvation. Methane oxidation was therefore confined to a very narrow zone where the oxygen concentration was less than 1 mg/liter (eg. 4.25 - 5 meters in Fig. 17). Within the zone of activity the oxidizers had access to nitrogen from nitrogen fixation and from ammonia which slowly diffused a steep concentration gradient from below (bottom water concentrations were ∿300 μM - NH_3 during summer stratification). Methane oxidation was absent below the zone of activity because of a lack of available electron acceptors.

Attempts to detect nitrogen fixation within the zone of methane oxidizing activity using $^{15}N_2$ (Flett unpublished data) were unsuccessful. It is likely that the rate of nitrogen fixation was below the limit of detection by this method (<.04 μg N/liter/hr). The more sensitive acetylene reduction technique could not be used within the zone of activity because acetylene has been found to inhibit methane oxidation (de Bont and Mulder, 1976) which likely starved the nitrogen fixation

process of energy.

Factors controlling the distribution of the methane oxidizers during periods of overturn (Fig. 18) can be explained with respect to the data in Fig. 15. Immediately after overturn concentrations of methane and oxygen were high throughout the water column (Fig. 18). The rate of methane oxidation was also very rapid throughout the column even though the oxygen concentration was high. This insensitivity to high oxygen concentration can be explained by the high concentration of NO_3 + NH_4^+ ($\sim$20 μM) which occurred throughout the water column at this time, the fixed nitrogen being swept up from the anoxic hypolimnion.

During the winter under ice-cover methane oxidation continued throughout the oxic portion of the water column at high oxygen (Fig. 19) and fixed nitrogen concentrations (NH_4^+ + NO_3 $\sim$40 μM). However during this time methane oxidation occurred most rapidly at oxygen concentrations of less than 1.0 mg/liter (Fig. 19). Thus even though the oxidizers were active at high oxygen concentration in the presence of adequate fixed nitrogen they still oxidized methane most effectively at oxygen concentrations of less than 1.0 mg/liter. This same phenomenon has also been observed during partial fall overturn (part 1 of this section).

Conclusions

When attempting to culture methane oxidizers from natural sources it is necessary to include in the medium lake water or sediment or soil extracts obtained from the same location as the sample. It is also necessary to simulate as closely as possible the optimal *in situ*

conditions of all other important parameters (eg. O_2 concentration). If these precautions had not been taken in our case the most important Lake 227 methane oxidizer could not have grown in culture due to a lack of an essential growth factor(s) and excessive oxygen concentration. Very often these precautions have not been included in culture methods described in the literature. As a result the methane oxidizing cultures obtained may be atypical representatives of the in situ methane oxidizing population.

Factors controlling the rates and distribution of methane oxidation in shield lakes as discussed in part 1 of this section must now be expanded to include the important controlling influence of the two possible nitrogen sources of the methane oxidizers (nitrogen fixation and DIN). It was concluded (part 1 of this section) that the presence or absence of thermal stratification was the most important controlling factor of methane oxidation rates since >95% of whole lake oxidation, on a yearly basis, occurred during periods of overturn and throughout the winter after water containing methane and oxygen had been mixed together. Although this conclusion is still valid it must now be recognized that these rapid whole lake methane oxidation rates would not have occurred if DIN (NH_4^+) had not been also swept up from the anoxic hypolimnion during overturn thus enabling the methane oxidizers to become oxygen insensitive. This is very important to whole lake metabolism under ice cover since methane oxidation can continue rapidly throughout the water column. This oxidation had been found to be a major contributor to the development of total anoxia in Lake 227 during winter (part 3 of this section) and it is a major consequence of eutrophication.

A second important controlling factor (part 1 of this section) was the sensitivity of the methane oxidizers to high oxygen concentration. This prevented epilimnetic methane oxidation during summer stratification and confined the oxidation to a narrow zone of activity usually within the metalimnion where oxygen concentrations were low. The reason for this oxygen sensitivity now appears to be the low epilimnetic DIN concentration and the inhibition of nitrogen fixation at high oxygen concentration. Thus epilimnetic oxidation ceased as a result of nitrogen limitation and oxidation was consequently confined to the narrow zone of low oxygen concentration in the metalimnion where the nitrogen fixation process was operative and NH_4^+ was available from the hypolimnion.

However, oxygen concentration also directly affects rates of methane oxidation since oxidation rates are most rapid at low oxygen concentration even during winter (Fig. 19) and periods of overturn (part 1 of this section) when DIN concentrations are high throughout the water column. Therefore the bacteria responsible for the methane oxidation in these lakes may be viewed as facultative microaerophiles since they can tolerate high oxygen concentrations under certain circumstances but operate optimally at oxygen concentrations of less than 1 mg/liter.

III Methane cycling in a eutrophic shield lake: effects on carbon cycling and whole lake metabolism.

Introduction

The previous two parts of this section were concerned mainly with the factors controlling the rates and distribution of methane oxidizing bacteria. With this background information in mind, as well as that of Cappenberg (1975) who described the factors controlling the rates and distribution of methane producing bacteria in sediments, the whole lake effects of methane cycling can now be considered.

Data is not available which quantitatively relates the amount of methane produced and oxidized in a lake to the total carbon budget. The possibility of carbon dioxide and bacterial cell material produced during methane oxidation acting as a carbon source for primary producers and secondary grazers also has not been examined. Thus, it can be concluded that the contribution of these two groups of bacteria to the carbon cycle of lakes has been generally ignored.

The effect of oxygen consumption by methane oxidizing bacteria on lake metabolism is also not well known although the possibility of these effects being important was suggested over 40 years ago (Rossolimo and Kusnezowa, 1934).

In this part *in situ* rates of methane production, oxidation, and evasion are presented on a seasonal and yearly basis for Lake 227. These rates are related to the carbon budget of the lake by comparison to total carbon input to the lake and to primary production. Data is also presented which relates the degree of oxygen depletion under ice-cover to the type of fall overturn.

Methods

Rates of limnetic methane oxidation to carbon dioxide and bacterial cell material and measurement of dissolved oxygen and methane concentrations were estimated as described in the General Methods Section.

Evasion of dissolved methane from the lake surface was estimated as recommended by Emerson et al. (1973). It was assumed that one meter and surface methane concentrations were identical and that the "stagnant boundary layer thickness" (ie. the theoretical thickness of the water layer through which dissolved gas must diffuse at the air-water interface) was 300 μ during summer stratification (as in Emerson et al. 1973) and 150 μ during fall overturn.

Whole lake rates of methane oxidation and the total mass of dissolved methane and oxygen in Lake 227 were calculated by multiplying the average rate or concentration over a depth interval by the depth interval volume of the lake (Brunskill and Schindler, 1971). These values were then summed to yield whole lake rates or masses. The corresponding areal values were calculated by dividing the totals by the surface area of the lake (5×10^4 m^2). This method assumes horizontal homogeneity which will be discussed later.

Rates of hypolimnetic methane production during summer stratification were calculated (as suggested by Rudd and Hamilton, 1975b), by monitoring the increase of dissolved hypolimnetic methane and the amount of methane consumption at the oxic-anoxic interface. These two values were summed and the total was divided by the surface area of anoxic hypolimnetic sediments to yield a methane production rate in a m Moles CH_4/m^2 hypolimnetic sediments/day.

Rates of hypolimnetic methane production during winter were calculated by dividing sediment surface area by the total mass of methane produced in the hypolimnion during that time period. This mass was determined by summing the amount of methane oxidized at the oxic-anoxic interface in the water column and the amount of methane that accumulated in the hypolimnion during the winter. For reasons discussed later the winters accumulation of hypolimnetic methane could not be estimated until early the next summer.

Since methane was not transferred from the hypolimnion to epilimnion during summer stratification and epilimnetic methane oxidation did not occur (see later), rates of methane production from epilimnetic sediments during summer were assumed to equal the rate of evasion of methane from the lake surface. Thus a production rate could be calculated by dividing the whole lake evasion rates by the surface area of the epilimnetic sediments yielding a rate in m Moles CH_4/m^2 epilimnetic sediments/day.

During winter under ice-cover, evasion rates were zero and again methane was not transferred from hypolimnion to epilimnion (see later). Thus total epilimnetic methane production equaled the rate of the epilimnetic rate of methane oxidation since epilimnetic methane concentrations did not increase substantially. A areal rate could then be calculated as above.

Tube Experiment:

Information concerning the site of methane production was obtained by sampling lake water isolated from the main water body in a large tube. A 4 m long polyethylene tube (cross-sectional area 625 cm^2) was fixed to one end of a rectangular plexiglass tube (25 cm x 25 cm x 5 m). The other end of the plexiglass tube was forced by divers into the sediments at 8 m depth. The plexiglass tube was used because polyethylene had been

found to be permeable to methane. The open end of polyethylene tube was fixed to a circular float at the surface of the lake such that a column of water from the surface to the sediment of the lake was enclosed. Five and one-half mCi of ^{14}C-Na HCO_3 was mixed into the surface water of the tube to begin the experiment.

To assess the fate and movement of this label, samples were pumped at meter intervals into all glass 50 cc syringes and preserved by increasing the pH to 11 with NaOH. Four ml of the sample was counted in a scintillation counter after addition of a dioxane based fluor (Schindler, 1966). Another 10 ml sub-sample was acidified and bubbled with air to remove any unincorporated ^{14}C-carbon dioxide. Four ml of this sub-sample was counted as before. The ^{14}C in the acidified sub-sample was considered to be fixed ^{14}C. The difference between the basic and acidic samples was considered to be unincorporated ^{14}C-carbon dioxide. The remainder of the sample was stripped and analyzed for ^{14}C-methane as described in the general methods section.

Results and Discussion

Site of Methane Production:

On June 22, 1973, the ^{14}C-Na HCO_3 was mixed mechanically to a depth of 0.5 m in the tube. During the next 35 days the ^{14}C label remained in an approximate 80:20 ratio in the particulate and ^{14}C-inorganic phases as it sedimented through the thermocline and into the anoxic hypolimnion. There was no detectable ^{14}C-methane production while the spike of ^{14}C activity descended through the hydrogen sulphide containing hypolimnion ^{14}C-methane production was first detected in a sediment-water slurry

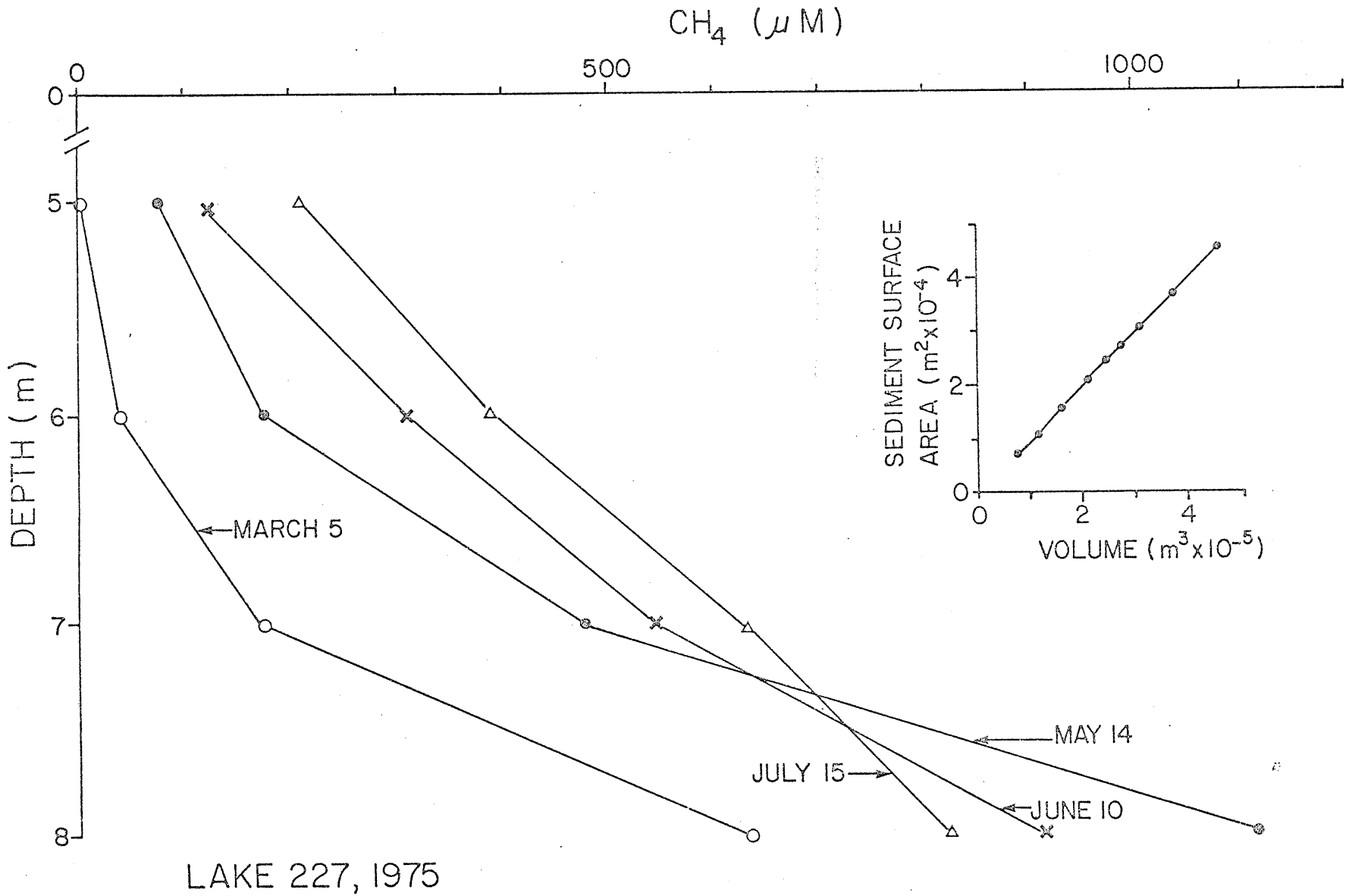
pumped from the sediment-water surface 9 days after the labelled material had reached the sediment surface. For the duration of the experiment (52 days) the amount of ^{14}C-methane within the tube increased most rapidly at sediment water interface indicating that all of the methane production was occurring in the sediments with subsequent diffusion into the hypolimnion.

Diffusion of Dissolved Methane During Periods of Stratification:

Rates of vertical diffusion in the hypolimnion and thermocline of Lake 227 were orders of magnitude slower than rates of horizontal mixing during summer stratification (P. Quay personal communication). Thus since methane was produced only the sediments of Lake 227, a linear relationship of depth versus methane concentration would be expected since there was a linear relationship between sediment surface area and lake volume, (inset Fig. 20) and because the lake was well mixed horizontally during this time of year (M. Stainton personal communication). In fact, such straight line summer hypolimnetic methane profiles have been observed. (Rudd and Hamilton 1975b and June and July profiles of Fig. 20) and can be used to estimate the total mass of dissolved hypolimnetic methane during summer stratification. In such a situation the dissolved methane in samples taken at the centre of the lake must have originated on the sides of the basin and diffused horizontally to the sampling point. Significant vertical diffusion up the water column from the sediment below was unlikely.

However, during the winter the lake was not well mixed horizontally. This resulted in edge effects with rapidly increasing methane concentrations near the sediments. This phenomenon is shown in Fig. 20 in which the

Fig. 20. The inset presents the linear relationship of sediment surface area versus the volume of Lake 227. The main figure presents anoxic hypolimnetic methane concentration profiles showing the gradual straightening of the profiles as a result of increased rates of horizontal mixing during summer.


CH4 (μM)
0
500
1000
0
5
6
7
8
DEPTH (m)
MARCH 5
MAY 14
JULY 15
JUNE 10
LAKE 227, 1975
SEDIMENT SURFACE AREA (m2x10-4)
VOLUME (m3x10-5)
0
2
4
0
2
4

hypolimnetic methane concentration profiles became progressively more curvlinear toward the end of the winter and then straightened early in the summer as a result of increased rates of horizontal diffusion in the hypolimnion. Apparently this incomplete horizontal mixing resulted in an overestimation of dissolved hypolimnetic methane as shown in the apparent but not real reduction in hypolimnetic methane just after ice-out in 1974 and 1975 (June 4, 1974 and May 8, 1975 anoxic CH_4 data Fig. 3a). Therefore the winter profiles could not be used to estimate the total mass of dissolved hypolimnetic methane and methane production rates. Instead the winter's accumulation of methane was estimated early the next summer after the profile had straightened. This method of estimation was possible because Lake 227 circulated to a depth of only 4-5 m after ice-out. Thus the winter's accumulation of dissolved methane remained in the hypolimnion.

Dynamics of Methane Cycling:

Sixty profiles of rates of methane oxidation in Lake 227 were obtained over a 26 month period. These data are presented as an isopleth and as whole lake rates in Fig. 21a and b. The total mass of dissolved methane in the oxygenated portion of the water column (oxic methane) for the same time period is shown in Fig. 22a. When the data is available amounts of dissolved methane in the deoxygenated hypolimnion (anoxic methane) are also presented. Methane evasion rates from the lake surface throughout the 26 month period are given in Fig. 22b.

Examining Figs. 21 and 22 together reveals much about the dynamics of methane cycling in Lake 227. During summer stratification (May - Sept. 1973, 74, 75), the very slow rate of vertical diffusion through

Fig. 21a. An isopleth of methane oxidation rates (μ moles/L/hr) in Lake 227 during a 26 month period. The stippled areas are zones where methane oxidation was absent. The depth of zero oxygen concentration closely followed the lower depth of zero methane oxidation. The solid circles on the horizontal axis represent sampling dates.

21b. Whole lake rates of methane oxidation during the same time period. These values may be converted to areal rates by dividing by 5×10^4 m^2.

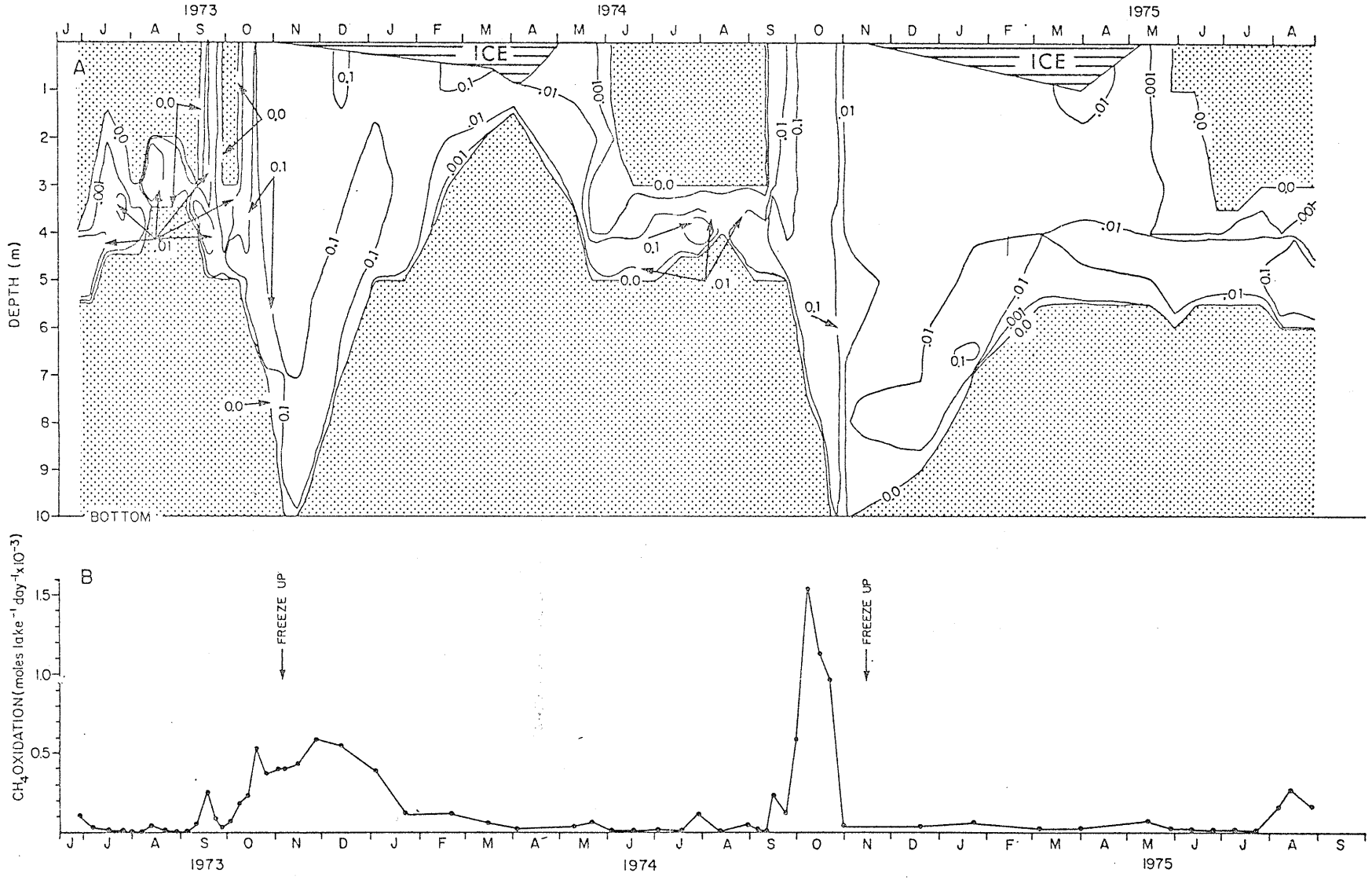
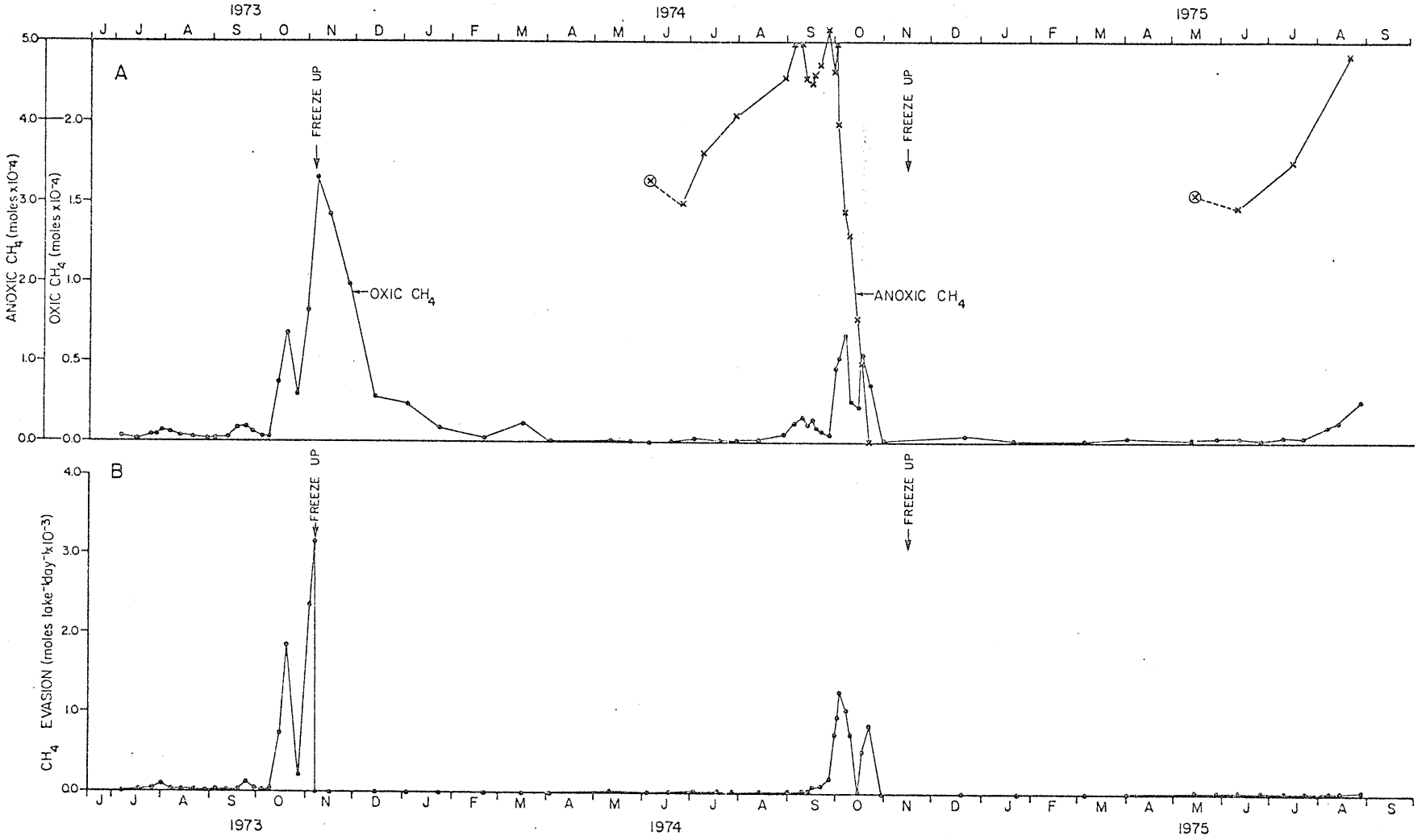

1973
1974
1975
J J A S O N D J F M A M J J A S O N D J F M A M J J A
ICE
ICE
A
DEPTH (m)
BOTTOM
B
FREEZE UP
FREEZE UP
CH4 OXIDATION (moles lake-1 day-1 x 10-3)

Fig. 22a. The quantities of dissolved methane in oxygenated water (oxic methane) and deoxygenated hypolimnetic water (anoxic methane) during a 26 month period.

22b. The rate of methane evasion from the lake surface during the same time period.


1973
1974
1975
J J A S O N D J F M A M J J A S O N D J F M A M J J A S
A
FREEZE UP
FREEZE UP
OXIC CH4
ANOXIC CH4
ANOXIC CH4 (moles x10-4)
OXIC CH4 (moles x10-4)
5.0
4.0
3.0
2.0
1.0
0.0
2.0
1.5
1.0
0.5
0.0
B
FREEZE UP
FREEZE UP
CH4 EVASION (moles lake-1day-1x10-3)
4.0
3.0
2.0
1.0
0.0
J J A S O N D J F M A M J J A S O N D J F M A M J J A S
1973
1974
1975

the thermocline virtually sealed off the anoxic hypolimnion from the rest of the lake (P. Quay personal communication). Therefore the mass of anoxic hypolimnetic methane progressively accumulated during the summer (Fig. 22a) with a large proportion (> 90%) of the methane produced during this time still present in the hypolimnion at overturn.

The small amount of methane that did diffuse vertically up to the oxic - anoxic interface within the thermocline during the summer was immediately and completely consumed by a narrow zone of methane oxidizing bacteria present at this interface Fig. 21a and Fig. 23. Since the rates of vertical diffusion were very slow, there was very little dissolved methane present in oxygenated water (oxic CH_4 data of Fig. 22a). Thus whole lake rates of methane oxidation were low (Fig. 21b) because methane oxidation occurred only in the presence of oxygen.[1]

Dissolved epilimnetic methane ($\sim$3 μM, Fig. 23) is believed to have originated from the littoral sediments since there was no transfer of dissolved methane from hypolimnion to epilimnion and because bubbling was not observed. This methane is considered to be that which was produced a few centimeters below the surface of the epilimnetic sediments and was "missed" by a narrow band of methane oxidizers known to be active at the sediment water interface as it diffused away from the sediments.

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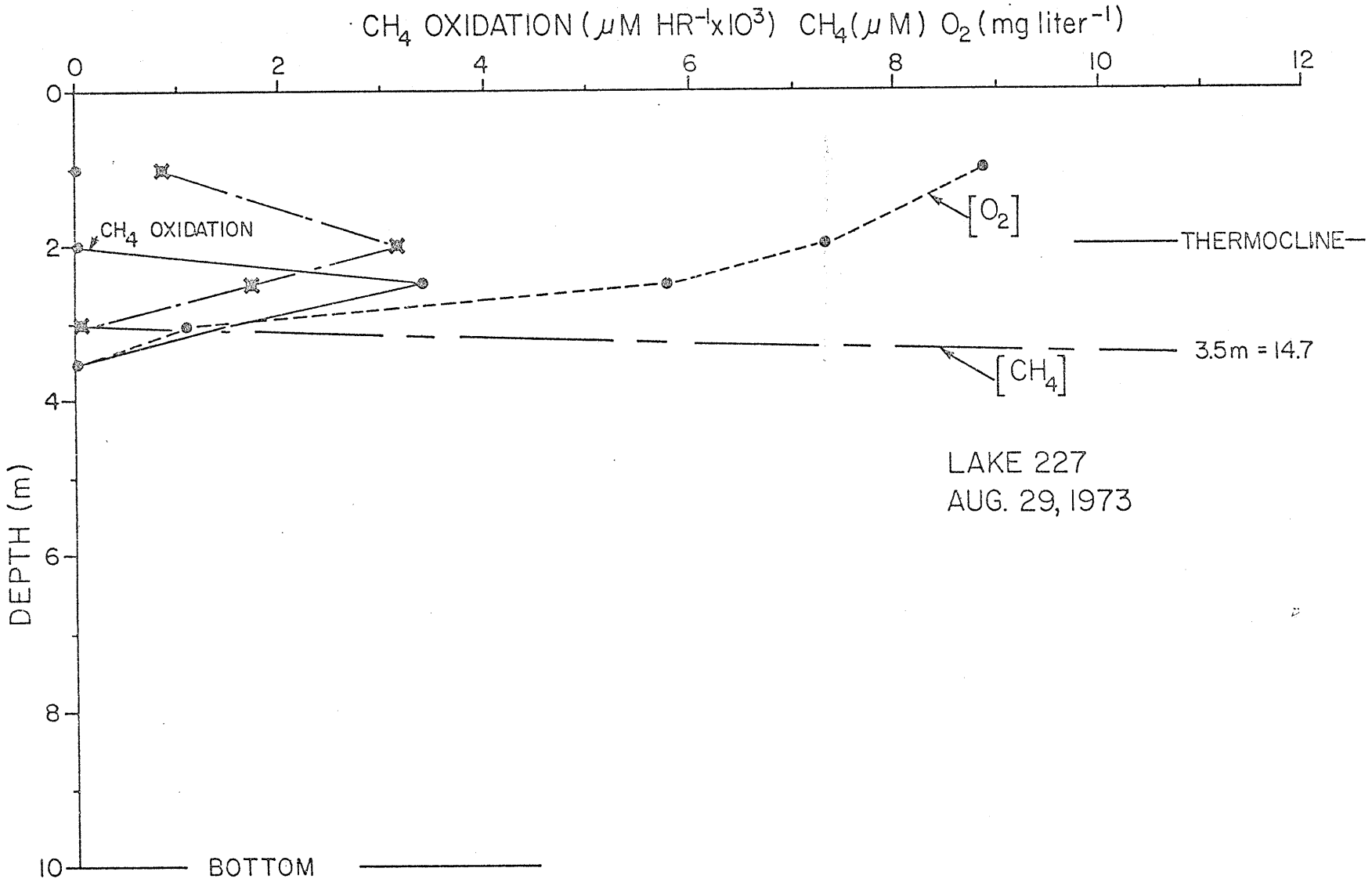
[1]The small amount of oxidation which occurred occasionally below the apparent zero oxygen concentration is believed to have occurred at oxygen concentrations below the limit of detection of the Winkler method. No evidence of the utilization of electron acceptors other than oxygen (eg. $SO_4^{=}$ and NO_3^-) could be obtained.

Fig. 23. A typical summer methane oxidation profile showing the very low methane concentrations within the zone of methane oxidizing activity at the oxic-anoxic interface and the higher epilimnetic methane concentrations as a result of an absence of methane oxidizing activity.


CH_4 OXIDATION (μM HR^{-1}x10^3) CH_4(μM) O_2 (mg liter^{-1})
0
2
4
6
8
10
12
DEPTH (m)
CH_4 OXIDATION
$[O_2]$
THERMOCLINE
3.5m = 14.7
$[CH_4]$
LAKE 227
AUG. 29, 1973
BOTTOM

The dissolved epilimnetic methane was not oxidized during the summer (Fig. 21a) despite the presence of high oxygen concentrations. This was because the epilimnetic methane oxidizers were nitrogen limited during summer due to the low in situ dissolved inorganic nitrogen concentrations and because their alternative nitrogen source, nitrogen dixation, was inoperative at the high epilimnetic oxygen concentrations (part 2 of this section). Consequently the epilimnetic methane slowly degassed to the atmosphere (Fig. 22b).

With the onset of fall overturn even a slight mixing of the water column (eg. Sept. 10-15, 1973) (Fig. 21a) was sufficient to significantly disturb the stable condition maintained through the summer. The injection of hypolimnetic water containing high concentrations of methane (Fig. 22a) and ammonia into the overlying oxygenated water, rendered the methane oxidizers oxygen insensitive (part 2 of this section), enabling methane oxidation to occur throughout the mixed portion of the water column (Fig. 21a) at increased whole lake rates (Fig. 21b). The rate of methane evasion was also increased (Fig. 22b) since epilimnetic water contained increased amounts of methane (Fig. 22a). These mixing effects were quickly reversed when circulation ceased (eg. Sept. 15-27, 1973 of Fig. 21 and 22).

As overturn progressed (Oct. 1973, 74) rapid rates of methane oxidation occurred over a progressively larger proportion of the water column (Fig. 21a) resulting in increased whole lake rates of methane oxidation (Fig. 21b). On a yearly basis greater than 90% of the methane oxidation occurred during and just after fall overturn. Rates of methane evasion (Fig. 22b) were directly controlled by surface water methane concentrations

which were in turn related to the in situ rates of methane oxidation. For example, during overturn 1974 evasion rates were lower than during overturn 1973 (Fig. 22b) likely because the high rates of methane oxidation (Fig. 21b) consumed the methane almost as quickly as it was swept up fromthe hypolimnion. This resulted in lower amounts of oxic methane (Fig. 22a) and consequently lower rates of evasion as compared to overturn 1973.

The sequence of events during fall overturn ended in one of two ways. Either all the methane was removed from the lake water by oxidation and evasion to the atmosphere before freeze-up (overturn 1974, Fig. 22a and b) or large quantities of oxic methane were trapped in the lake when freeze-up terminated methane evasion (overturn 1973, Fig. 22a and b).

In the latter case this resulted in high whole lake rates of methane oxidation throughout the oxygenated portion of the water column early the next winter (Nov. 6 1973 - Jan 22, 1974 of Fig. 21a and b). The much lower rates of methane oxidation during the winter of 1974 - 75 (Fig. 21b) were probably as a result of the very low amounts of dissolved oxic methane (Fig. 22a) since virtually all of the methane had either been oxidized or evaded to the atmosphere during overturn.

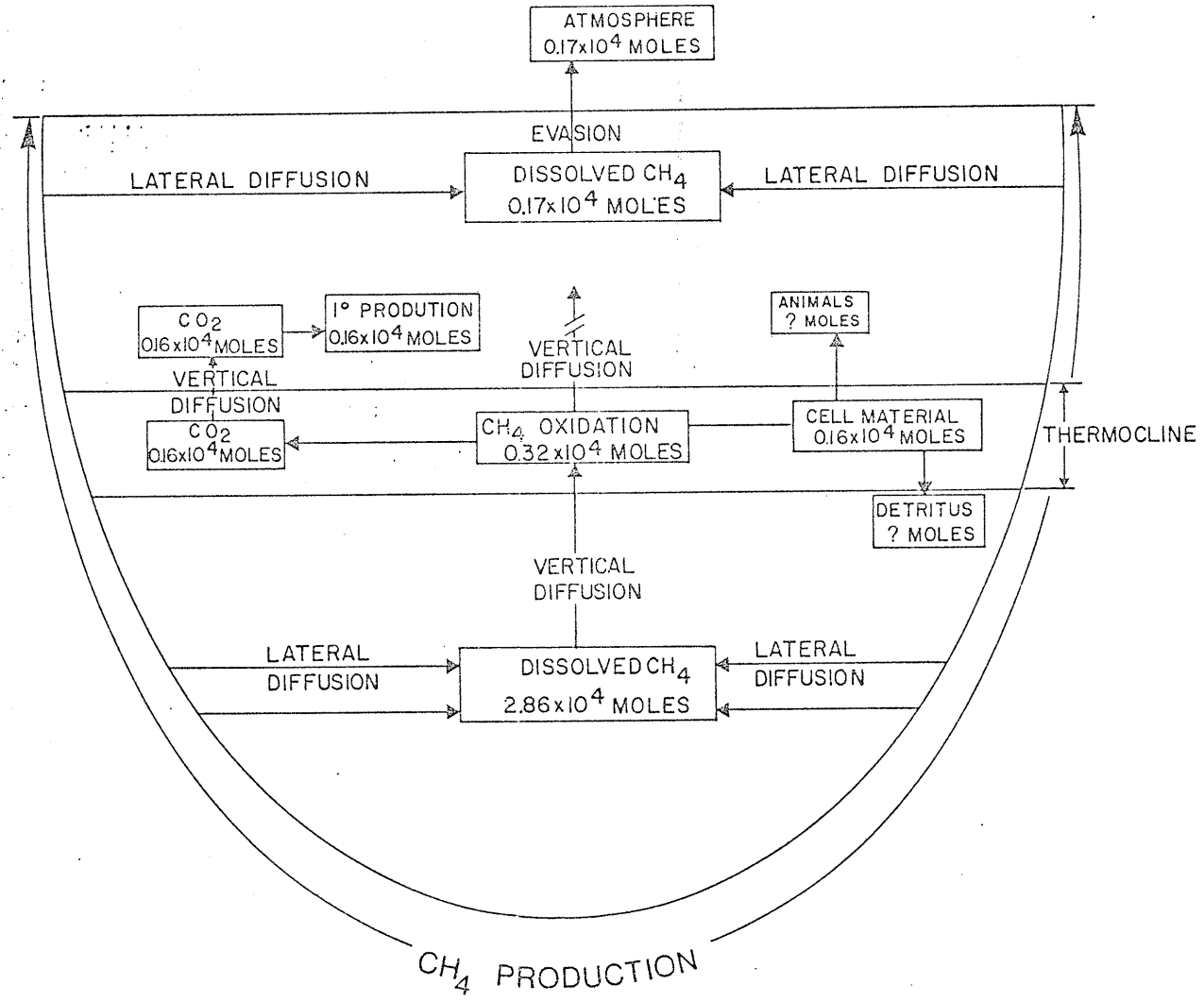
Since Lake 227 mixes only to a depth of 4 - 5 meters after ice-out only a small peak in methane oxidizing activity would be expected at that time. Slight increases in whole lake methane oxidation rates were observed early in May of 1974 and 1975 (Fig. 21b) which were probably the tail end of a small peak in activity that was missed as a result of inaccessibility of the lake during break-up.

Lake 227 Methane Budget:

Summer - During the summer of 1974 (May 21 - August 30) 2.68×10^4 moles (10.8 mM/m^2 hypolimnetic sediment/day) of methane were produced in the hypolimnion of Lake 227. Of this, 0.32×10^4 moles were consumed by methane oxidizing bacteria at the oxic-anoxic interface (Fig. 24). The total amount of hypolimnetic methane at the end of this period was 4.55×10^4 moles (not 2.68×10^4 moles) because hypolimnetic methane had been accumulating since the previous fall turnover. Thus an amount of methane equal to only 7% of the methane present in the hypolimnion at the beginning of fall overturn had diffused vertically to the oxic-anoxic interface within the thermocline during the previous summer. Since virtually all this methane was consumed by the methane oxidizers (Fig. 23) there was no movement of the methane from hypolimnion to epilimnion during summer stratification. The oxidized methane was converted in a 50:50 ratio to carbon dioxide and bacterial cell material (Fig. 24). An undetermined portion of the bacterial cell material would probably have been consumed by animal grazers, the remainder becoming detritus. The carbon dioxide produced by the oxidizers would have continued to diffuse up the carbon dioxide concentration gradient into the epilimnion. There it was completely consumed by primary producers since there was a very large epilimnetic carbon dioxide deficit in Lake 227 during the summer of 1974 as a result of intense phytoplankton blooms (D.W. Schindler personal communication). Since there was no epilimnetic methane oxidation during the summer in Lake 227 (part 1 and 2 of this section and Fig 21a), all the methane diffusing from the epilimnetic sediments (0.17×10^4 moles or 0.8m Moles/m^2 epilimnetic sediment/day) degassed to the atmosphere at an average rate of 0.34 m M/m^2 lake surface/day.

Fig. 24. Production, oxidation and evasion of dissolved methane during summer stratification.

SUMMER STRATIFICATION
1974
LAKE 227



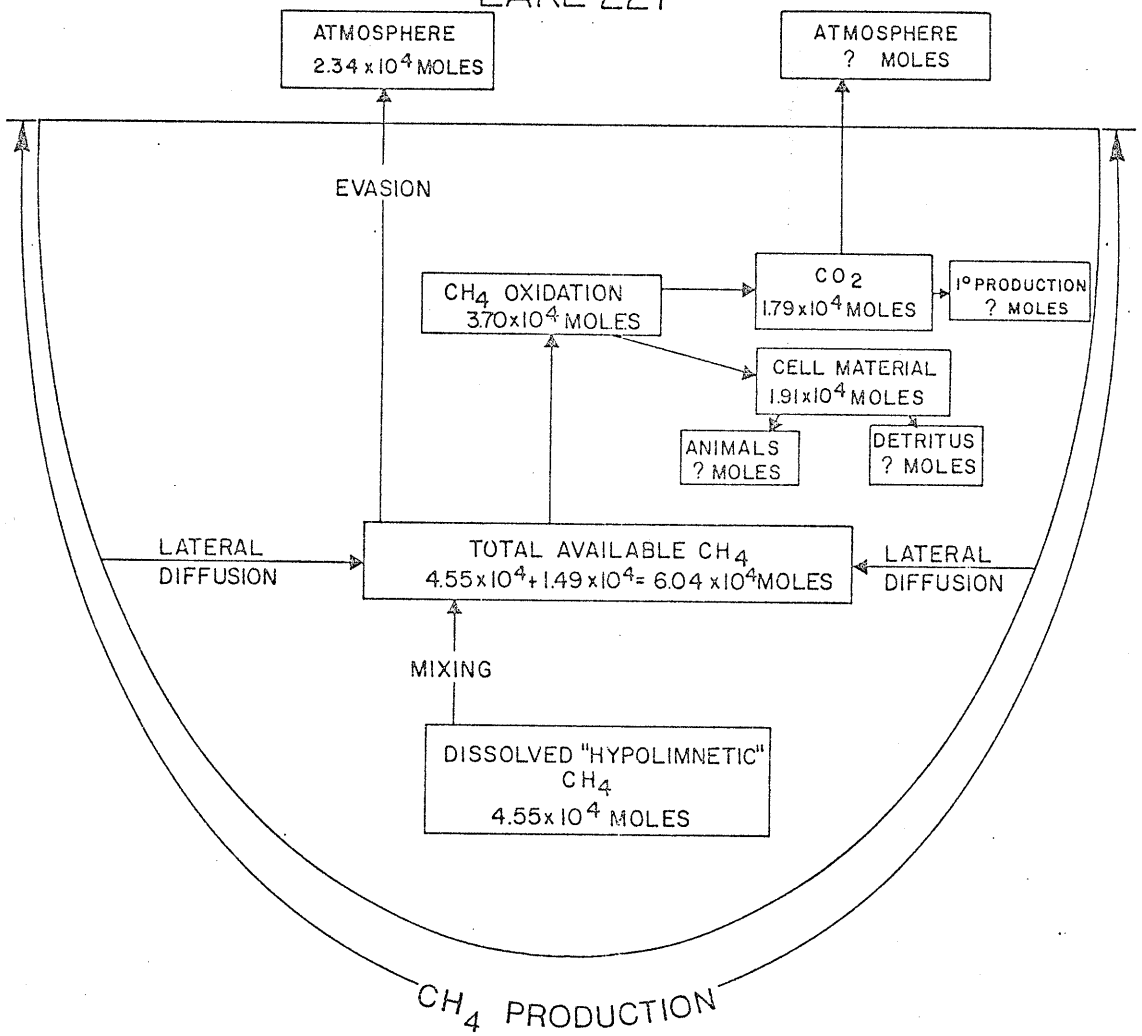
Two factors probably contributed to the slower rates of methane production by the oxic epilimnetic sediments as compared to the anoxic hypolimnetic sediments (0.8 vs 10.8 mMoles/m^2 sediment/day). In the presence of oxygen a large proportion of the organic carbon degraded at the epilimnetic sediment-water interface would have been converted to carbon dioxide instead of methane. Also, methane produced anoxically deep in the sediments would have been largely consumed before it entered the epilimnion by the methane oxidizers active at the sediment-water interface.

Fall Overturn - During fall overturn (Fig. 25) the 4.55 x 10^4 moles of hypolimnetic methane were mixed into the overlying oxygenated water. During this period another 1.49 x 10^4 moles of methane were added to the lake from both the oxic and anoxic sediments (4.8m Mole/m^2 total lake sediment/day). Thus a total of 6.04 x 10^4 moles methane were available for methane oxidation during the overturn. Of this, 61% was oxidized in a 50:50 ratio to bacterial cell material and carbon dioxide and 39% escaped to the atmosphere (Fig. 25) at an average evasion rate of 7.6 x 10^4m Mole/m^2 lake surface/day. However, this evasion rate was highly variable (Fig. 22b) ranging from 1 - 23.4 m M/m^2 lake surface/day. Most of the carbon dioxide produced during overturn must have evaded to the atmosphere since Lake 227 is supersaturated with carbon dioxide during this time of the year (Schindler 1972) and because primary production was reduced at this time of year (Fee).

Winter - During the winter (Nov. 15, 1974 - May 7, 1975) an anoxic hypolimnion developed with the hypolimnetic sediments producing methane at approximately the same rate as during the summer (2.47 x 10^4 moles or 12.1 m M/m^2 hypolimnetic sediment/day). The similarity of summer

Fig. 25. Production, oxidation and evasion of dissolved methane during fall overturn.

FALL OVERTURN
1974
LAKE 227



and winter rates is probably a reflection of the constant rate of substrate supply to the methane procuders. Since the supply ultimately depends on primary production, which is seasonal, the link between methane production and primary production must be remote (see next section).

Again, only a small portion (9.3%) of the dissolved hypolimnetic methane diffused vertically to the oxic-anoxic interface (Fig. 26). Most of the remainder was still present in the hypolimnion at the beginning of the next fall overturn (Sept. 1975). At the oxic-anoxic interface the methane was consumed by the methane oxidation and as before, converted approximately 50:50 to carbon dioxide and bacterial cell material (Fig. 26). In this case the carbon dioxide continued to diffuse up the concentration gradient into the epilimnion where it accumulated until ice-out since degassing was prevented by ice cover and primary production was almost totally inhibited by snow on the ice (E.J. Fee, personal communication). Almost all of the methane diffusing from the oxic sediments during the winter was consumed by methane oxidizers active in the epilimnion (Fig. 26). It will be shown in a later section that during certain winters epilimnetic methane oxidation has been found to be a major contributor to the development of total lake anoxia under the ice.

Participation of Methane in the Carbon Cycle of Lake 227:

The contribution of methane to the Lake 227 carbon cycle is summarized in Table 1. The total amount of methane leaving both oxic and anoxic sediments during the year (ice-out 1974 to ice-out 1975) amounted to 17.8 gC/m^2 lake surface. During this time 11.8 gC/m^2 lake surface was oxidized within the lake in a 50:50 ratio to carbon dioxide

Fig. 26. Production, oxidation and evasion of dissolved methane under winter ice.

WINTER 1974-1975
LAKE 227

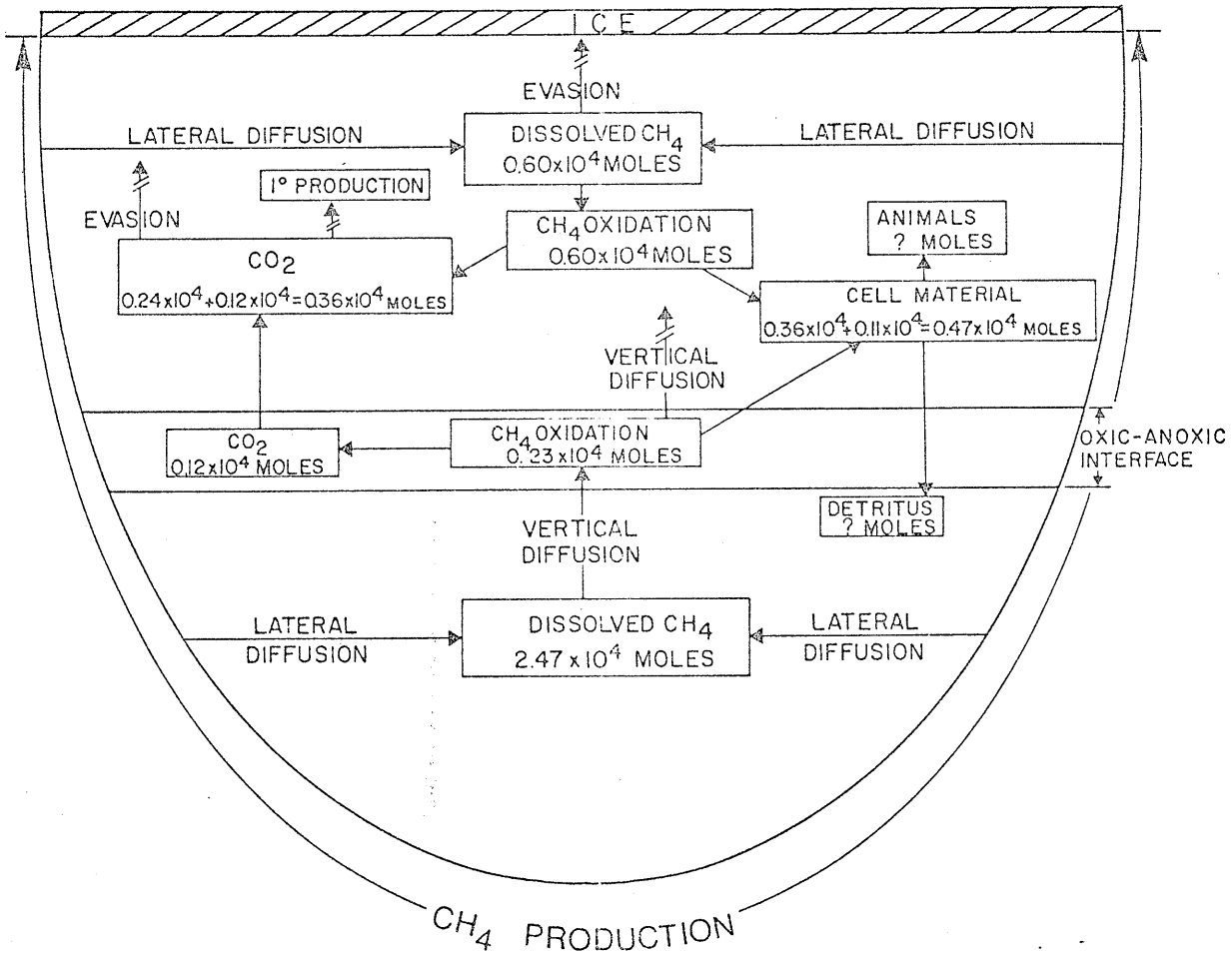


Table 1. The participation of methane in the carbon cycle of Lake 227 in comparison to primary production and total carbon input. The primary production and total carbon input although presented on a yearly basis occurred mostly during the summer of 1974. The period considered for methane production and oxidation was chosen to include the succeeding winter since carbon fixed during the previous summer would be re-appearing as dissolved methane.

		gC m^2 lake surface $^{-1}$
1.	CH_4 production (ice-out '74 to ice-out '75)	17.8
2.	Total CH_4 oxidation (ice-out '74 to ice-out '75)	11.8
3.	CH_4 oxidizing bacterial production (ice-out '74 to ice-out '75)	5.9
4.	CO_2 production by CH_4 oxidizers.	5.9
5.	Primary production (1974) [1]	272.4
6.	Total carbon input (1974) [2]	32.5

[1] E.J. Fee personal communication

[2] D.W. Schindler personal communication

and bacterial cell material. Total primary production during 1974 (which virtually all occurred in the ice-free season) amounted to 272.4 gC/m^2 lake surface. Therefore, methane oxidation was not a major carbon dioxide source for primary production not only because only 5.9 gC/m^2 lake surface of carbon dioxide were produced but also because greater than 90% of the carbon dioxide production from methane occurred during fall overturn and throughout the winter (Fig. 21b) when photosynthesis was practically non-existent. This supports the conclusion of Schindler et al. (1972) and Schindler and Fee (1973) that the major source of carbon dioxide for primary production during the summer in Lake 227 was atmospheric.

Similarly, the amount of bacterial cell material produced by the methane oxidizers was insignificant, compared to primary production (Table 1). Therefore methane oxidizing bacteria are probably not an important source of fixed carbon for most animal grazers. A possible exception to this occurred early in the winter of 1973-74 when methane oxidation rates were high (Fig. 21a,b). At that time bacterial cell material produced during methane oxidation may have been a significant food source for grazers since primary production rates were very low under the ice.

However, from another point of view the contribution of the methane cycle to the annual carbon cycle was much more important. Total carbon input (comprised of inputs from runoff and the atmosphere) amounted to 32.5 gC/m^2 lake surface (D.W. Schindler personal communication, Table 1). Methane production regenerated 55% of this total carbon input from ice-out 1974 to ice-out 1975. Sixty-five percent of this methane (or 36% of the total carbon input) was oxidized by bacterial methane

oxidation during the same time period. Therefore there appears to be a very significant methane contribution to carbon cycle of Lake 227 in terms of total carbon input into the lake. The large difference between the amount of carbon fixed by primary producers and the amount of carbon entering the lake (Table 1) may be explained by recycling of carbon a number of times within the epilimnion during the summer by aerobic decomposition.

Effects of Methane Oxidation on Dissolved Oxygen Concentration:

The most important effects of methane cycling on whole lake metabolism resulted from the consumption of dissolved oxygen by the methane oxidizing bacteria. During the winter of 1973 - 74 this caused the lake to become totally anoxic (Table 2).

Whether or not the lake became totally anoxic during the winter was determined by the type of fall overturn. If the lake circulated for a few weeks before freeze-up (as in Oct. 1974, Fig. 21 and 22) virtually all of the methane was either oxidized or degassed to the atmosphere (the sediments were also probably largely emptied of dissolved methane, Line 1 of Table 2). In this case although methane oxidation still contributed substantially to oxygen consumption during the winter of 1974-75, (Line 4, Table 2), a large amount of oxygen was still present in the lake just before ice-out (Line 6, Table 2) and epilimnetic oxygen concentrations remained above 5 mg/1.

On the other hand during the fall of 1973 the lake froze almost immediately after overturn (Nov. 1973, Fig. 3) trapping a large quantity of dissolved methane under the ice (Line 1, Table 2). Since the methane

Table 2. The contribution of methane oxidizing activity under the ice to the disappearance of dissolved oxygen.

	Total Lake 227 Values in Moles	Winter of 1973-74	Winter of 1974-75
1.	Dissolved CH_4 at freeze-up	1.66×10^4	11.0
2.	Dissolved O_2 at freeze-up	5.53×10^4	5.86×10^4
3.	Percent O_2 consumed by (1)	45%*	0.03%
4.	CH_4 oxidation under ice	4.06×10^4	0.82×10^4
5.	Percent of O_2 consumed by (4)	110%	21%
6.	Dissolved O_2 one month before ice-out.	0.0	1.4×10^4

* An O_2:CH_4 ratio of 1.5:1 was used to calculate the amount of oxygen consumed during methane oxidation. This ratio was based on Klass et al. (1969) but the amount of oxygen consumed per mole of methane was increased proportionately to account for the 1:1 cell material to carbon dioxide production of Lake 227 methane oxidizers as compared to the 1.7:1 ratio of cell material to carbon dioxide production in Klass et al. (1969).

oxidizers were not sensitive to high oxygen concentration under the ice because the high dissolved inorganic nitrogen concentrations replaced nitrogen fixation as nitrogen source (part 2 of this section), methane oxidation proceeded rapidly (Fig. 21a and b) throughout the oxygenated portion of the water column. The oxidation of this methane plus that which diffused from the sediments during the winter (a total of 4.06×10^4 moles, Table 2) was sufficient to cause total lake anoxia (line 6, Table 2). As a result the suffication of many fish, zooplankton and zoobenthos were observed (I. Davies personal communication).

It is very desirable to prevent the winter kill of fishes and invertebrates in many lakes. If methane oxidation is a general cause of lake anoxia during winter this could be accomplished by adding an inhibitor of methane oxidation to the lake just after freeze-up (92.2% of the methane oxidation and 96.6% of the oxygen disappeared from the lake within 77 days of freeze-up). Acetylene, a known inhibitor of methane oxidation (Whittenbury et al. 1974, de Bont 1976) is presently under consideration. As an inhibitor it has several attractive characteristics: it is very soluable in water, very small amounts are required (approximately 0.1 μM), it is non-toxic at this low concentration, it is not readily metabolized by the methane oxidizers (J.A.M. de Bont personal communication) and it is both inexpensive and convenient to add to the lake as a solid (calcium carbide).

GENERAL CONCLUSIONS

A method was not available to sensitively, precisely and rapidly monitor rates of methane oxidation. Therefore a ^{14}C tracer method was developed (which has also been described by Rudd et al. 1974 and Rudd and Hamilton, 1975a). Using this method several possible factors controlling in situ rates of methane oxidation were examined (Rudd and Hamilton 1975a and part 1 of the results section). It was concluded that the two most important controlling factors were the presence or absence of thermal stratification which regulated the movement of the dissolved substrates of the methane oxidizer and the sensitivity of the methane oxidizers to high oxygen concentrations especially during summer. Methane concentration was of less importance and control of whole lake methane oxidation by temperature and pH was rarely if ever noted.

In part 2 of the results section (Rudd et al. 1976) an explanation of the sensitivity of the methane oxidizers to high oxygen concentration was presented. It was concluded that this sensitivity during summer was related to their dependence upon an oxygen sensitive nitrogen fixation process. The oxidizers were less sensitive to high oxygen concentration during overturn and throughout the winter because increased concentration of dissolved inorganic nitrogen replaced nitrogen fixation as a nitrogen source. With this information factors controlling the rates and distribution of methane oxidizers in lake 227 were explained on a yearly basis.

In part 3 of the results section (Rudd and Hamilton in preparation) the whole lake methane cycle was related to whole lake metabolism. The amounts of methane produced oxidized and degassed to the atmosphere

on a seasonal basis were compared to amounts of carbon fixed by primary production and to the total amount of carbon entering the lake. It was concluded that methane cycling had a very significant input into the carbon cycle in that an amount of carbon equal to 55% of total carbon input into Lake 227 was regenerated during one year. Sixty-five percent of this methane (or 36% of total carbon input) was oxidized in the lake by methane oxidizing bacteria. The amounts of carbon dioxide and bacterial cell material produced by methane oxidizing bacteria were small in comparison to the primary productivity of the lake. Therefore methane oxidation would not have been a significant carbon dioxide source for primary producers or a food source for most grazers.

During one winter the oxidation of large quantities of methane trapped under the ice caused Lake 227 to become totally anoxic. This resulted in the suffocation of many fish, zooplankton and zoobenthos.

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