HETEROTROPHIC UTILIZATION OF SUCROSE IN AN ARTIFICIALLY ENRICHED LAKE

A Thesis Submitted to

the Faculty of Graduate Studies and Research

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Barbara Mary Thompson

December, 1972

ACKNOWLEDGEMENTS

The author takes this opportunity to thank Dr. R.D. Hamilton, Adjunct Professor of the Department of Microbiology, University of Manitoba, for advice and encouragement throughout the course of this investigation, and for assistance in the preparation of this manuscript.

Thanks are also due to Dr. D.W. Schindler of the Freshwater Institute for making available facilities at the Fisheries Research Board of Canada Experimental Lakes Area, and for providing access to data pertaining to this study.

I would also like to thank Dr. E.J. Fee and Mr. R.V. Schmidt for assistance with computer aspects.

ABSTRACT

Utilization of sucrose by heterotrophic micro-organisms was measured in a small lake enriched with 5.54 g C/m^2/year. Heterotrophic activity exhibited fluctuations representing the damped oscillations of a disturbed steady state, stabilizing three months after the commencement of enrichment. By late summer reduction of the weekly addition of sucrose to low levels was accomplished within a day by the increased microbial activity resulting from rapid growth of heterotrophic micro-organisms. Heterotrophic conversion (pre-enrichment) of sucrose to particulate carbon in the epilimnion between July and October 1971 was 2-17% of primary production. 25-35% of total sucrose utilized was converted to free carbon dioxide.

TO MY FATHER

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

List of Tables and Figures Cont'd.

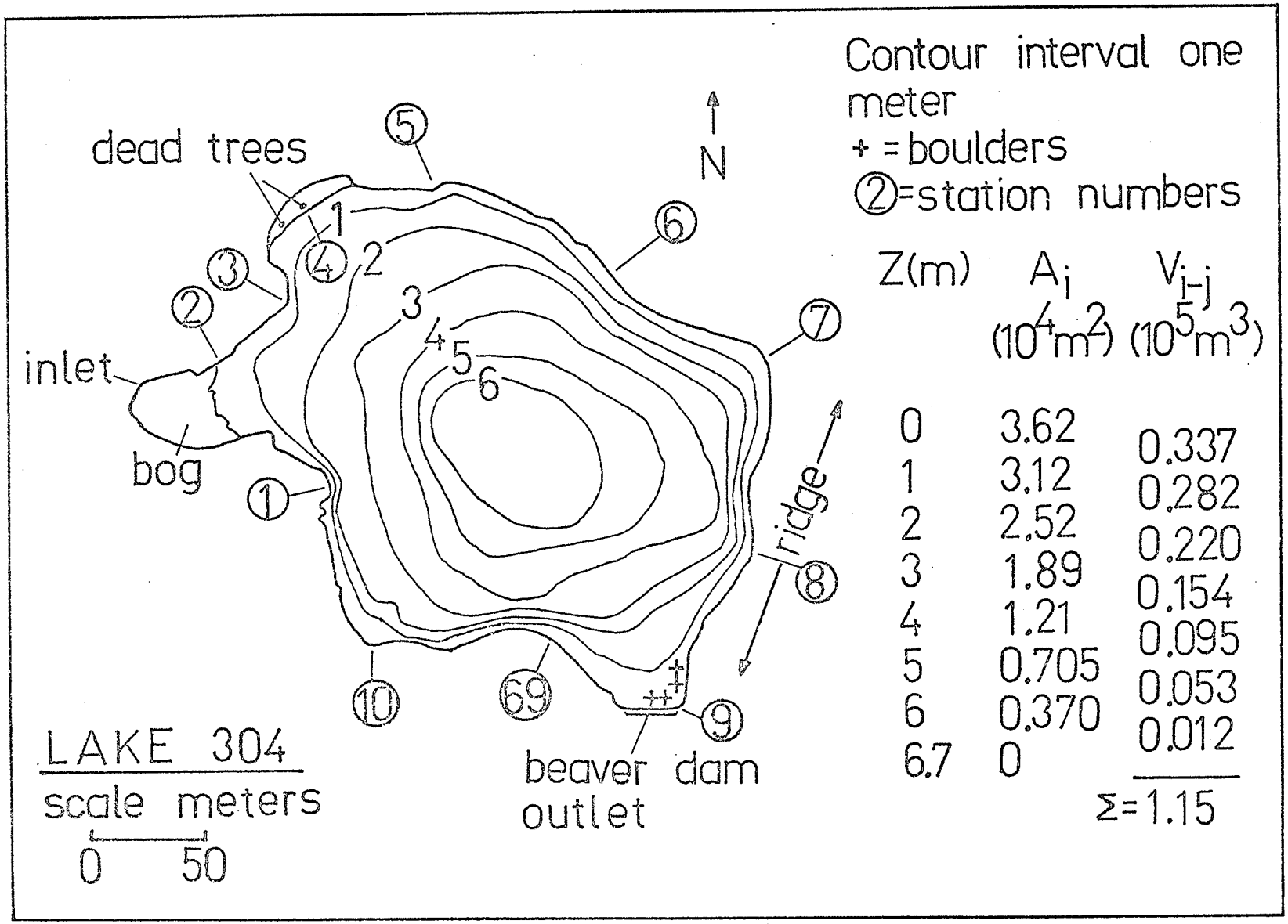
INTRODUCTION

Fig. 1. Bathymetric chart of Lake 304 (2).

INTRODUCTION

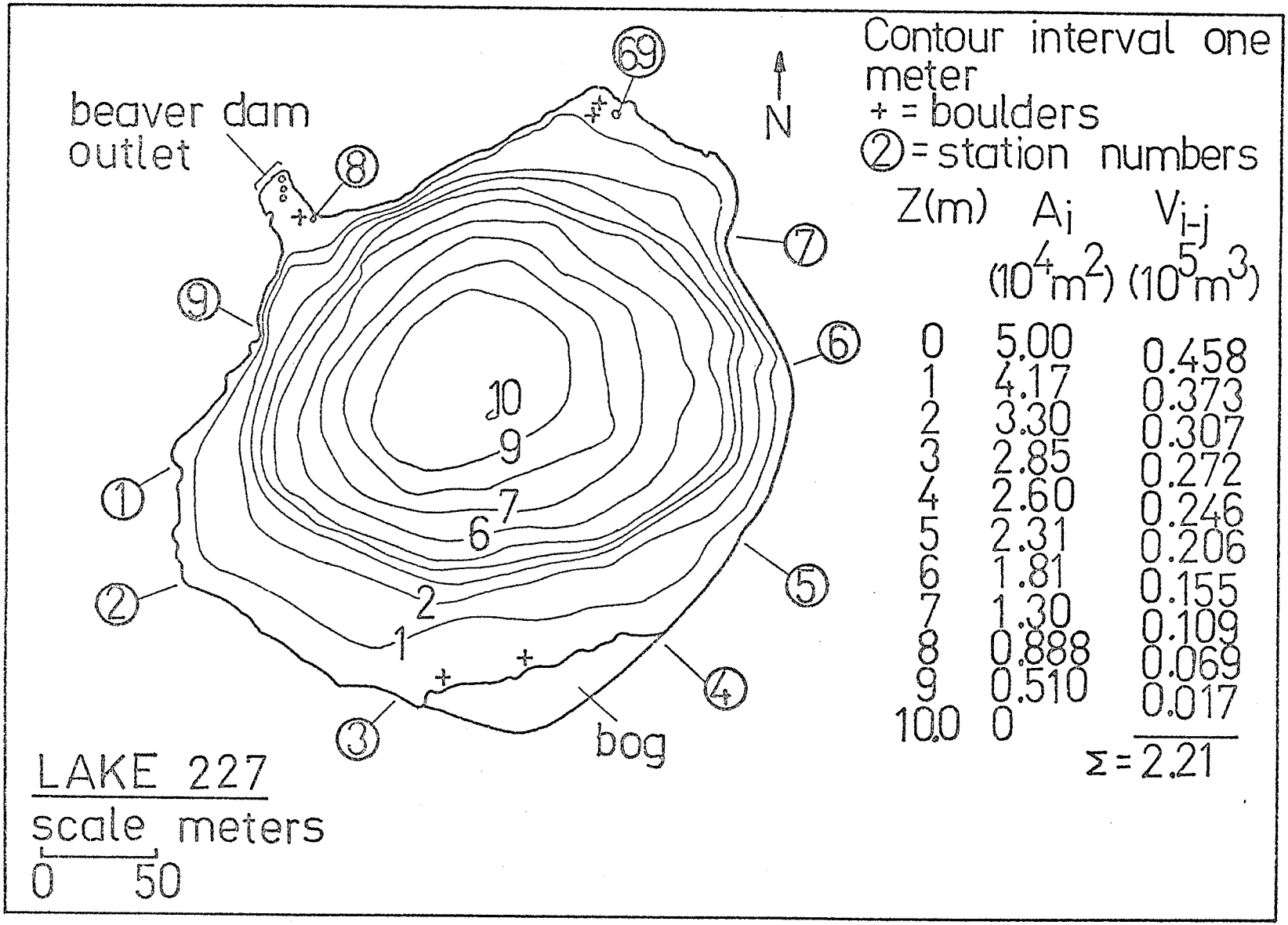
The following study was carried out in the Fisheries Research Board of Canada Experimental Lakes Area, northwest Ontario (1). The Experimental Lakes Area, situated about 56 km southeast of the town of Kenora, exhibits typical characteristics of boreal forest underlain by Precambrian Shield. Bedrock is acid granite, overlain in places by thin glacial drift (2). Soils are sparse on hilltops and slopes, with 10-30 cm of brunisols in low-lying areas, more in areas underlain with glacial till. The area supports fire-controlled, subclimax forest, chiefly of jack pine, black spruce, trembling aspen and white birch. Lake basins in this area were formed during the recession of glacial Lake Agassiz which covered the area approximately 10,000 years ago (3). Drainage is into Hudson's Bay via Lake Winnipeg and the Nelson River.

Research at the Experimental Lakes Area is directed towards understanding the problem of eutrophication and developing remedial measures. As part of this programme, Lake 304 was enriched with carbon, nitrogen and phosphorus as sucrose, ammonium chloride and orthophosphoric acid, commencing in the spring of 1971. Lake 304 (Fig. 1) is a small naturally oligotrophic lake, area 3.62 hectares, maximum depth 6.7 meters, situated 15 km west of the field laboratory. Its selection for a whole lake enrichment experiment was based on accessibility, small size, simple morphometry and thermal stratification during the summer. During 1971 enrichment proceeded at weekly intervals from May 26 to October 13 inclusive; during 1972 from May 24 to October 4 inclusive. Loading rates were 0.4 g phosphorus, 5.18 g nitrogen and 5.54 g carbon per m^2 per year. These levels of enrichment were chosen independently



Z(m)	A_i ($10^4 m^2$)	V_{i-j} ($10^5 m^3$)
0	3.62	0.337
1	3.12	0.282
2	2.52	0.220
3	1.89	0.154
4	1.21	0.095
5	0.705	0.053
6	0.370	0.012
6.7	0	
		$\Sigma = 1.15$

Fig. 2. Bathymetric chart of Lake 227 (2).



Z(m)	A_i ($10^4 m^2$)	V_{i-j} ($10^5 m^3$)
0	5.00	0.458
1	4.17	0.373
2	3.30	0.307
3	2.85	0.272
4	2.60	0.246
5	2.31	0.206
6	1.81	0.155
7	1.30	0.109
8	0.888	0.069
9	0.510	0.017
10.0	0	
		$\Sigma = 2.21$

of the study reported here. The resulting C:N:P ratio of 14:13:1 is not the same as that in sewage. Nitrogen and phosphorus levels were selected after a consideration of Vollenweider's correlation between loading rate and trophic state of water bodies (4); the N:P ratio is similar to that of plants. Carbon additions were chosen to produce the N:C ratio obtained in sewage. Sucrose additions were equivalent to 84 µg carbon per liter per week, or 200 µg sucrose per liter per week over the whole lake volume.

Lake 227, (Fig. 2) which was sampled on two occasions, is situated in the Experimental Lakes Area 5 km northeast of the field laboratory. Its origin and morphology are similar to those of Lake 304, though it is slightly larger (area 5 hectares, maximum depth 10 meters). Lake 227 has been enriched with nitrogen as sodium nitrate and phosphorus as ortho-phosphoric acid for the last three years, and can now be termed eutrophic.

The importance of heterotrophic micro-organisms in the utilization of added sucrose was investigated with the radiochemical method first used by Parsons and Strickland (5) and developed further by Wright and Hobbie (6). Mineralization of sucrose was determined using a slight modification of the radiorespirometric methods of Williams and Askew (7), Hobbie and Crawford (8) and Harrison et al. (9). Part of the thesis deals with problems encountered when using these techniques. Sucrose-carbon incorporated by heterotrophic processes in Lake 304 was compared with carbon fixed by photosynthesis.

HISTORICAL

HISTORICAL

Early studies of the role of bacteria in lakes and oceans were largely confined to estimates of their numbers (10, 11, 12). The cultural and direct microscopical methods used led to widely differing results and contributed little to an assessment of microbial activity. The need to estimate microbial contributions to nutrient cycling led Parsons and Strickland (5) to develop a radiocarbon technique measuring what they called the "relative heterotrophic potential" of natural populations in seawater. This technique is analagous to that of Steemann-Nielsen (13) for measuring primary productivity.

Obligate heterotrophs are organisms which rely on organic compounds as a source of energy and of carbon incorporated into cellular material. By "relative heterotrophic potential" Parsons and Strickland (5) meant the potential ability of a naturally-occurring population of hetero-trophic micro-organisms to incorporate soluble carbon into particulate material; they recognized that this may not be the same as *in situ* incorporation of these nutrients.

Parsons and Strickland (5) measured incorporation of radioactivity into particulate matter after incubating seawater samples with uniformly labelled ^{14}C-glucose or ^{14}C-acetate. Relative heterotrophic potential, or velocity of substrate incorporation under experimental conditions, was obtained from:

$$\left(\begin{array}{c}\text{Fraction of total radioactivity}\\ \text{incorporated}\end{array}\right) \times \left(\begin{array}{c}\text{Substrate}\\ \text{added}\end{array}\right) / \left(\begin{array}{c}\text{Incubation}\\ \text{time}\end{array}\right)$$

By using a range of substrate concentrations, substrate incorporation was found to be amenable to analysis according to Michaelis-Menten enzyme kinetics (14).

Wright and Hobbie (6) extended this kinetic approach of Parsons and Strickland (5). The Michaelis-Menten equation relating velocity of substrate conversion into particulate carbon to substrate concentration is as follows:

$$v = \frac{V_{max} S}{K + S} \qquad (1)$$

where v is the velocity of reaction as substrate concentration S, V_{max} the maximum velocity attainable (sites saturated) and K (the saturation constant) that substrate concentration at which $v = \frac{V_{max}}{2}$.

One of the more rigorous linear transformations of (1) (15) is a modification of the Lineweaver-Burke equation:

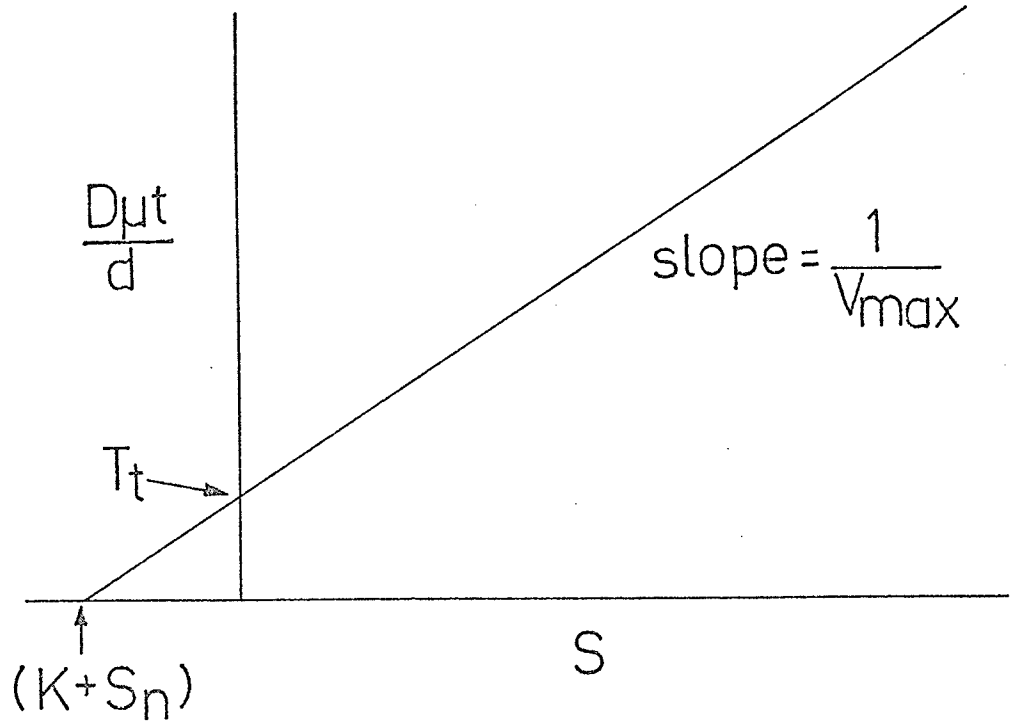
$$\frac{S}{v} = \frac{1}{V_{max}} \cdot S + \frac{K}{V_{max}} \qquad (2)$$

Relative heterotrophic potential is equivalent to v so:

$$\frac{D\mu t}{d} = \frac{1}{V_{max}} \cdot S + \frac{(K + S_n)}{V_{max}} \qquad (3)$$

where D is the dpm from 1 μCi ^{14}C, μ the μCi added to that portion of the incubated sample which is filtered, t the incubation time in hours, d the dpm utilized per volume filtered, S the substrate added and Sn the naturally occurring substrate concentration, both in μg/l. K is the transport constant for the microbial population, and is a measure of the affinity of the system for substrate. A low K indicates ability to utilize low substrate concentrations and vice versa. Kinetic parameters obtained from a plot of $\frac{D\mu t}{d}$ against S are shown in Fig. 3.

Fig. 3. Kinetic parameters obtained from a plot of $\frac{D\mu t}{d}$ against S.



T_t = turnover time of naturally occurring substrate

K = transport constant

S_n = concentration of naturally occurring substrate

V_{max} = maximum velocity attainable

v_n = velocity of substrate utilization when $S = S_n$

Kinetics of the Michaelis-Menten type are widely applicable to the growth of micro-organisms (16) including protozoa (17), algae and bacteria. However, Michaelis-Menten enzyme kinetics, if strictly interpreted, can only be applied to single enzyme-single substrate systems. Criticism of the heterotrophic potential approach was inevitable and the validity of applying enzyme kinetics to a mixed population of microbial heterotrophs has been questioned (18, 19) as erratic uptake patterns have been obtained in oceanic waters of low productivity (20, 21).

To test the validity of the basic assumption of Parsons and Strickland (5), Williams (22) fitted random values of K and V_{max} into a computer model of a natural population. The data so generated, when incorporated into an S/v against S plot, resulted in overestimation of turnover time by about 25% and a functional value of K for the population which is not a simple mean of the individual values of K. An accurate estimate of V_{max} could be obtained from the linear part of the curve at high values of S. The author concluded that the simple kinetic equation may be used to describe utilization of substrate by natural microbial populations "providing that its weaknesses are recognized".

Heterotrophic micro-organisms obtain energy from a portion of the soluble organic material utilized, thus liberating CO_2. A further portion may be released as organic by-products and the remainder incorporated into cellular material. Therefore in order to assay utilization, d in equation (3) should be corrected to $(d_i + d_r + d_e)$ where D_i is dpm incorporated, d_r dpm respired as $^{14}CO_2$ and d_e dpm excreted as soluble organics. No estimate of the latter term has been attempted in conjunction with uptake of nutrients by micro-organisms in natural waters; however,

the importance of taking respiration into account when measuring substrate utilization was soon recognized. A study of the mineralization of specifically labelled glucose by a marine pseudomonad (23) showed that 10 to 38% of added label remained in the cells while 8 to 74% was respired, depending upon the position of the label.

The magnitude of mineralization led Hobbie and Crawford (8) to develop a closed system technique whereby $^{14}CO_2$ is absorbed by a basic amine on a filter paper wick after acidifying the incubated water sample. Investigation of heterotrophic utilization of uniformly labelled glucose, acetate and amino acids showed that respiration as a percentage of total uptake varied between 8% (arginine) and 60% (aspartic acid). Mineralization of glucose by micro-organisms in seawater (7) and sediments (9) has also been measured.

Taking $^{14}CO_2$ respiration into account raises the value of V_{max} based on uptake alone and decreases turnover time. Theoretically, no change in $(K + S_n)$ should occur, though Hobbie and Crawford (8) noted a small decrease due to respiration. Mineralization of dissolved organics provides an obvious source of CO_2 for phytoplankton photosynthesis; it is not known to what extent $^{14}CO_2$ is incorporated into algal biomass during incubation.

The flux of nutrients through a microbial population is perhaps the most useful measure of heterotrophic activity (24, 25). Flux is the velocity of substrate utilization (v_n) at the concentration of substrate occurring naturally. Accurate measurement of flux requires knowledge of substrate uptake, mineralization and natural substrate concentration; v_n is calculated from S_n/T_t.

Measurement of nutrient concentrations in natural waters presents difficulties, as many organics are present at microgram per liter levels. Biological, chemical, physical and enzymatic techniques have been devised to overcome this problem. A bioassay micro-organism is one of known K. S_n can be derived from $(K + S_n)$ obtained on incubation of the micro-organism with filter-sterilized water sample and varying concentrations of the substrate subject to analysis. Hobbie and Wright (26) used a bacterial isolate from Lake Erken, Sweden, to assay glucose in fresh water. However, after culturing on rich media for several months, the K of the organism increased. Vaccaro and Jannasch (18) used Achromobacter aquamarinus to detect glucose down to 4×10^{-8}M. Dilution of natural water samples to obtain $(K + S_n)$, $(K + \frac{S_n}{2})$, and hence K and S_n, has shown mixed success (27, 28). The accuracy of this technique depends upon the quality of the diluent. Alternatively, dilution may disturb heterotrophic activity by reducing the concentration of other nutrients and ions and by altering the physical relationship between the diverse components which make up the natural microflora.

Of the several methods for physically isolating trace organics prior to chemical analysis, chromatography has proved to be the most useful. Levels of amino acids in seawater have been obtained by ligand-exchange chromatography and automatic ion exchange chromatography (29, 30). Concentrations of free sugars in lake-water and sediments have been estimated by evaporation and paper chromatography (31, 32).

Determination of organic compounds at low concentrations by enzymatic techniques shows promise, as a carefully chosen reaction sequence can combine both specificity and sensitivity. Glucose in seawater has been determined by the glucose oxidase system following charcoal extraction and evaporation (33). A method of glucose determination requiring no prior concentration of water sample utilizes the fluorescent reduced form of the dye resazurin, and is sensitive down to 10^{-8}M (34,35).

A consideration of microbial heterotrophy cannot neglect the possibility of algal heterotrophy. Wright and Hobbie (27) found uptake of glucose and acetate at concentrations greater than 500 µg/l to be due to diffusion. Using pure cultures of bacteria and algae isolated from fresh water, they concluded that bacteria remove low concentrations of substrate from water via specific transport systems, while algae can remove higher concentrations by passive diffusion. Algal heterotrophy has been demonstrated at substrate concentrations between one and six orders of magnitude greater than concentrations recorded from natural waters (36, 37, 38).

Sloan and Strickland (37) commenting on studies with four marine algae, neatly summed present knowledge by concluding: "heterotrophic survival of these and probably most other algae in the open ocean would be impossible unless they were in contact with a high concentration of substrate in the form of particulate matter".

The choice of substrates used in kinetic analyses of heterotrophic micro-organisms is often questioned. Glucose and acetate were used initially as they were the only common metabolites available at suitably high specific activity. Amino acids, glucose and acetate have

been used without determining their relative importance as phytoplankton excretory products. Glycollic acid, an early product of photosynthesis, is released from cultured freshwater and marine algae in amounts representing up to 38% of carbon fixed during photosynthesis (39, 40); excretion varies for different species of algae. Wright (41), recognizing that phytoplankton loss may be heterotroph gain, measured uptake of glycollic acid in a slightly eutrophic lake. He found active bacterial uptake of this compound only in the upper layers of the lake; uptake and production of glycollic acid were of the same order of magnitude. However, low and erratic uptake of glycollic acid by heterotrophic micro-organisms in the open ocean has been reported (21). A similar approach is to observe heterotrophic utilization of labelled algal extracellar dissolved organic carbon (42) or labelled hydrolysate of algae (43). These paths of investigation promise understanding of phytoplankton-heterotroph relationships and flux of photosynthetically fixed carbon.

In conclusion, it is necessary to survey the results of previous enrichments of fresh water with sucrose. Carbon enrichment has often been used to increase fish production in ponds and streams (44). A most notable study involved addition of sucrose to a section of an experimental trout stream in the Willamette River Basin, Oregon (45). The enriched section received about 55 kg sucrose/m^2/week for one year. Food consumption by trout was doubled and fish production increased sevenfold as compared to unenriched sections. Sucrose enrichment led to the growth of the slime bacterium Sphaerotilus natans which provided food and habitat for chironomid larvae, an important fish

food. As far as is known, no study of the role of heterotrophic microorganisms in the utilization of sucrose in artificially enriched water has been performed. The enrichment of Lake 304 afforded us such an opportunity.

MATERIALS AND METHODS

MATERIALS AND METHODS

Uniformly labelled ^{14}C-sucrose (600 mCi/mM) and ^{14}C-D-glucose (288 mCi/mM) were obtained from Amersham/Searle. Uniformly labelled ^{14}C-D-fructose (121 mCi/mM) was obtained from New England Nuclear. Solutions of 1 µCi and 5 µCI per ml were made up in Super Q water (Millipore^{R}), filter sterilized through washed autoclaved 0.22 µ pore-size Millipore HA filters, and aseptically dispensed into sterile 10 ml glass ampoules (previously washed with conc. H_2SO_4 and rinsed with distilled water) which were sealed and stored at -20^{o}C. Unlabelled sucrose, D-glucose and D-fructose solutions were prepared in a similar manner in concentrations which would give a final concentration of 5 and 10 µg per liter when 0.1 ml was added to 50 ml of lake water. A sterile solution of 200 µg sucrose per ml was prepared for a laboratory enrichment experiment.

Samples were taken with either a 2 l Van Dorn sampler or a battery-powered peristaltic pump (S igmamotor Inc., Middleport, N.Y.). The inside of the Van Dorn was scrubbed with 99% isopropanol (Fisher Scientific) and thoroughly rinsed with sterile lake water. The peristaltic pump was fitted with autoclaved silicone and sur gical rubber tubing. Discrete water samples (by Van Dorn) and integrated water samples (by peristaltic pump) were taken above and below the thermocline, sampling from the surface down so as not to unduly disturb the water column. Predatory zooplankton were removed by filtering the water through an autoclaved 100µ stainless steel mesh which was rinsed with a portion of each water sample prior to filtering. Samples were collected in autoclaved polypropylene bottles, first rinsed out with a

small amount of sample water. Sampling took place regularly 1-21 hours prior to enrichment, at the center station in Lake 304; sampling of Lake 227 took place at the station representing maximum depth. All samples were transported to the laboratory in a back-pack and processed within 1-1.5 hours of sampling.

In the laboratory, 50 ml volumes of each sample were dispensed into acid-washed (10% HCl) distilled water-rinsed autoclaved 125 ml glass bottles. Labelled sucrose, 0.1 μCi, (equivalent to a final concentration of 1.14 μg sucrose/l of lake water) was added to each bottle, followed by 5 or 10 μg sucrose/l increments of unlabelled sucrose up to 40 or 50 μg sucrose/l. Blanks were prepared at two or three concentrations and fixed immediately after addition of sucrose with neutral 40% formalin at a final concentration of 4%. A similar procedure was employed when studying D-glucose and D-fructose uptake.

Bottles were incubated in the dark for 1.5 - 3 hr. at $\pm$ 2-3°C of *in situ* temperature. Incubations were performed in the dark in an attempt to prevent photosynthetic assimilation of $^{14}CO_2$. The incubation time was chosen after consulting the relevant literature; it was also shown that in this study uptake of substrate remained linear over the incubation time. Five ml from each bottle was filtered at a vacuum of less than 300 mm. Hg through a 0.45 μ pore size Millipore HA filter and rinsed with 2 x 5 ml distilled water. The water was just drawn through the filter each time and the filter was then placed in a glass or polythene scintillation vial and dissolved in a dioxane-based fluor, that of either Bray (46) or Schindler (47) (Appendix III). On several occasions different volumes of water sample were filtered and the filtration error calculated (Appendix II).

The above incubation procedure was repeated with a series of acid-washed, sterile 100 ml serum bottles which were sealed with

rubber serum stoppers immediately after addition of substrate. These samples were used for the collection of respired $^{14}CO_2$, as described in Appendix I.

Radioactivity in particulate matter or CO_2 was measured by liquid scintillation on a Picker Liquimat 220 liquid scintillation counter. Samples were counted to at least 1028 counts (standard deviation 4%). Counts per minute were converted to disintegrations per minute by the channels ratio technique utilizing an internal standard. The counting efficiency was between 55 and 75%. Three or four scintillation vials containing 0.05 or 0.1 ml of radioactive substrate in dioxane based fluor were prepared and counted during each experiment in order to determine the µCi added to the incubated samples.

D-glucose and D-fructose Utilization in Lake 304

On two pre-enrichment dates in 1971 (August 31 and September 15) measurements were made of uptake of glucose and fructose by heterotrophic micro-organisms in the epilimnion of Lake 304. Sampling, incubation and filtering procedures were as already described; unlabelled D-glucose and D-fructose were added up to 60 µg/l.

Sucrose Utilization in the Presence of D-glucose and D-fructose, Lake 304

On September 22, 1971, uptake of sucrose by heterotrophic micro-organisms in the epilimnion of Lake 304 was measured in the presence of up to 20 µg/l D-glucose or D-fructose. 0.1 µCi of ^{14}C-sucrose (U) and six increments of unlabelled sucrose up to 50 µg/l were added to seven sets of 50 ml aliquots of epilimnetic water. One set of bottles contained sucrose additions only; the other sets contained, respectively, 5, 10 and 20 µg. D-glucose/l and 5, 10 and 20 µg D-fructose/l.

Utilization of D-glucose and Sucrose in Lake 227

D-glucose and sucrose utilization in the epilimnion of Lake 227 were compared on two occasions in 1972. On July 22 uptake of D-glucose and sucrose at 1 m were measured. Up to 40 µg/l unlabelled substrate was added to the incubated samples; incubation time was 2 hr. Uptake and respiration of glucose and sucrose at two depths in the epilimnion of Lake 227 (0.5 m and 2.0 m) were measured on July 26. Additions of unlabelled substrate up to 25 µg/l were made, and the incubation time was 1.5 hours. Further processing to determine uptake of substrate proceeded as already described in this section; mineralization of substrate was measured as described in Appendix I.

Laboratory Enrichment Experiment, Lake 304

Enrichment with sucrose in the laboratory was performed on a 6 l integrated epilimnetic sample in a sterile 8 l glass carboy covered with aluminium foil to cut out the light. The sample was continually stirred with a sterile stirring bar and bubbled with sterile air for five minutes every half hour (except between 12 and 24 hours) via a sterile pipette inserted through a stopper in the neck of the carboy. Fifty ml subsamples were dispensed through an outlet in the base of the carboy into acid-washed sterile 125 ml glass bottles, after discarding the first 50 ml. Before addition of sucrose to the carboy, 0.1 µCi of ^{14}C-sucrose (U) was added to a series of 50 ml. subsamples, followed by increments of unlabelled sucrose up to 40 µg/l. The carboy and incubated samples were kept at room temperature (21^{o}C) which was close to *in situ* temperature (20.58^{o}C); all incubations took place in the dark and lasted 1 hour. Filtration and further processing was as previously described. Uptake only was measured.

At zero time (t = 0) 200 µg/l unlabelled sucrose was added to the carboy, this value representing the concentration immediately after enrichment assuming mixing through the whole lake volume. At t = 0.25, 1.5, 3.0, 4.5, 6.0, 9.0, 12.0 and 24.0 hours, a series of 50 ml subsamples was taken, to each of which was added 1 µCi ^{14}C sucrose and unlabelled sucrose to total 12, 22, 32 and 42 µg/l; further processing to determine uptake was performed as already described.

Results were calculated assuming that at 5 = 0.25 hr., S = 200 + x where x is µg sucrose/l added to the subsample. When velocities of sucrose uptake were calculated they were found to be very close in value, so the average was used as a velocity over the next time interval to calculate the amount of sucrose remaining at the next sampling time.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Statistics of Sampling

To explore the reproducibility of sampling and incubation techniques, replicate samples were taken at a single station in Lake 304 and incubated with identical concentrations of labelled and unlabelled sucrose. The variation in dpm incorporated into heterotrophic microorganisms was expressed by means of the standard deviation as a percentage of the mean. This statistical procedure was also applied to subsamples taken from the same sample bottle, and replicate 5 ml aliquots from the same incubated sample. Results are shown in Table 1.

Effect of neglecting Filtration Error

Changes in kinetic parameters obtained when taking filtration error (Appendix II) into account are shown in Table 2. Neglecting filtration error could lead to underestimation of $(K + S_n)$ and V_{max} by up to a factor of two, and overestimation of T_t by up to two thirds. Values for August 11, 1971 were obtained by applying the filtration correction at high substrate concentration only since a correction for low substrate additions was not available; the changes in kinetic parameters thus obtained are believed to be erroneous and have been disregarded.

Effect of neglecting Mineralization of Substrate

Table 3 indicates the changes in kinetic parameters expected when carbon dioxide production (Appendix I) is taken into account. The

TABLE 1

Statistics of Sampling

Standard deviation of dpm on filter per 5 ml of incubated sample expressed as a percentage of the mean.

	Replicate samples taken at same station	Replicate sub-samples taken from same sample bottle	Replicate 5 ml aliquots from same incubated sample
Standard deviation as % of mean	11.95, n[1] = 6	6.87, n = 4	1.38, n = 6
	15.41, n = 4	10.35, n = 5	
	11.76, n = 5		

[1]Denotes number of samples per experiment.

TABLE 2

Changes in Kinetic Parameters due to Filtration Error, Lake 304.

Date	Depth	Factor required to correct kinetic parameters obtained from uptake of substrate for the error due to filtration		
		$(K + S_n)$	V_{max}	T_t
4/8/71	Epilimnion	1.0	1.30	0.77
4/8/71	Hypolimnion	1.0	1.58	0.64
11/8/71	Epilimnion	4.28	2.26	1.91'
11/8/71	Hypolimnion	11.85	1.33	9.03'
17/8/71	Hypolimnion	1.97	2.05	0.95

'Filtration correction applied only to high substrate values.

TABLE 3

Changes in Kinetic Parameters when Mineralization of Substrate is taken into account, Lake 304 and Lake 227.

Date	Depth	Factor required to correct kinetic parameters obtained from uptake of substrate for mineralization of substrate.		
		$(K + Sn)$	V_{max}	T_t
24/8/71	Epilimnion	1.26	1.83	0.69
21/10/71	0-3 m.	10.32	2.51	7.55
21/10/71	3-6 m.	0.96	1.35	0.72
26/7/72	2 m.	1.04	1.33	0.77'
1/8/72	Epilimnion	0.67	1.18	0.57

' Lake 227, glucose utilization

values obtained on October 21, 1971 at 0-3 m are considered erroneous as $(K + S_n)$ for uptake only was abnormally low, possibly owing to the prescence of a large filtration error. Considering the remaining values, it can be seen that V_{max} is underestimated up to 1.83 times and T_t is overestimated as much as 0.57 times if one neglects mineralization of substrate. Values of $(K + S_n)$ corrected for mineralization vary between 0.67 and 1.26 times the uncorrected values.

These data indicate that the combined effect of correcting for filtration error and mineralization of substrate upon kinetic parameters derived from uptake of substrate alone would increase $(K + S_n)$ up to 2.5 times and V_{max} up to 3.8 times, while decreasing T_t by as much as 0.4 times.

Kinetic parameters

Kinetic parameters obtained from the analysis of the utilization of organic nutrients can be interpreted in relation to a population of micro-organisms. K represents the affinity of the micro-organisms for substrate. A low K indicates ability of the micro-organisms to utilize low concentrations of substrate, while a high K indicates that relatively high substrate levels must be present before such populations could become effective competitors for the substrate. Since $(K + S_n)$ is obtained from this treatment of heterotrophic activity, a large $(K + S_n)$ implies an unadapted population and/or high natural substrate levels, while a small $(K + S_n)$ represents an adapted population with a low concentration of natural substrate.

TABLE 4

Utilization of Sucrose in Lake 304, July 1971 to January 1972

Date	Depth	Measurement	$(K + S_n)$ µg/l	V_{max} µg/l/hr	T_t hours	Correlation* %
14/7/71	Hypolimnion	b	1.2	0.58	2.1	99
21/7/71	Hypolimnion	a	11	0.87	12	88
28/7/71	Epilimnion	a	63	9.1	6.9	84
4/8/71	Epilimnion	a	11	2.4	4.4	98
"	Epilimnion	b	11	3.1	3.4	98
"	Hypolimnion	a	5.1	0.31	16	99
"	Hypolimnion	b	5.1	0.49	10	99
11/8/71	Epilimnion	a	6.1	0.46	13	96
"	Epilimnion	b	26	1.0	25	96
"	Hypolimnion	a	0.26	0.39	0.66	99
"	Hypolimnion	b	3.1	0.52	6.0	97
17/8/71	Epilimnion	a	31	3.9	8.1	98
"	Hypolimnion	a	7.4	0.61	12	96
"	Hypolimnion	b	15	1.3	12	92
24/8/71	Epilimnion	a	7.1	1.2	5.9	99
"	Epilimnion	c	9.8	0.96	10	94
"	Epilimnion	d	9.0	2.2	4.1	98
"	Hypolimnion	a	5.5	0.35	16	97
31/8/71	Epilimnion	a	3.3	1.4	2.3	94
15/9/71	Epilimnion	a	8.7	1.0	8.6	91
"	Hypolimnion	a	0.65	0.13	5.0	99
22/9/71	Epilimnion	a	5.9	1.3	4.6	94
29/9/71	0-3 m	c	11	2.2	4.9	98
"	3-6 m	a	4.9	1.9	2.6	94
21/10/71	0-3 m	a	1.0	1.3	0.78	97
"	0-3 m	c	4.5	0.77	5.9	99
"	0-3 m	d	10	3.2	3.2	92
"	3-6 m	a	7.4	2.3	3.2	94
"	3-6 m	c	6.4	0.78	8.2	92
"	3-6 m	d	7.1	3.1	2.3	94
9/11/71	2 m	a	3.8	0.07	54	96
19/1/72	2 m	b	16	0.31	53	93

Table 4. Cont'd.

Measurement:

a Uptake, not corrected for filtration error

b Uptake, corrected for filtration error

c Mineralization

d Mineralization plus uptake, not corrected for filtration error

*This figure represents the % variation in S which is due to variation in S/v.

V_{max} gives information about the size of the microbial population (27). A small V_{max} indicates the presence of few micro-organisms capable of utilizing a particular substrate. A large V_{max} denotes a relatively larger number of such micro-organisms.

The value obtained for T_t is a composed of several factors. A long T_t may result from a small adapted population of micro-organisms in an environment containing high natural substrate levels, or a large or small unadapted population. Similarly a short turnover time is observed when either a small adapted population and low natural substrate concentration or a large adapted population are present.

Kinetic parameters representing heterotrophic activity in the epilimnion and hypolimnion of Lake 304 from July 1971 to January 1972 are shown in Table 4. The table also indicates changes in kinetic parameters obtained when filtration error and mineralization of sucrose are taken into account. Typical plots of v against S and S/v against S are shown in Figures 4 and 5.

Figures 6, 7 and 8 show the changes in V_{max}, T_t and $(K + S_n)$, respectively, of heterotrophic micro-organisms in the epilimnion and hypolimnion of Lake 304 between July 1971 and January 1972. The data shown in these figures represents incorporation of sucrose 1-21 hours prior to the weekly enrichment; filtration correction has been applied to the January data only since uncorrected data were not statistically reliable.

Values of V_{max} were higher in the epilimnion than in the hypolimnion until thermal stratification broke down in the fall. This

Fig. 4. v against S, Lake 304, August 4, 1971.

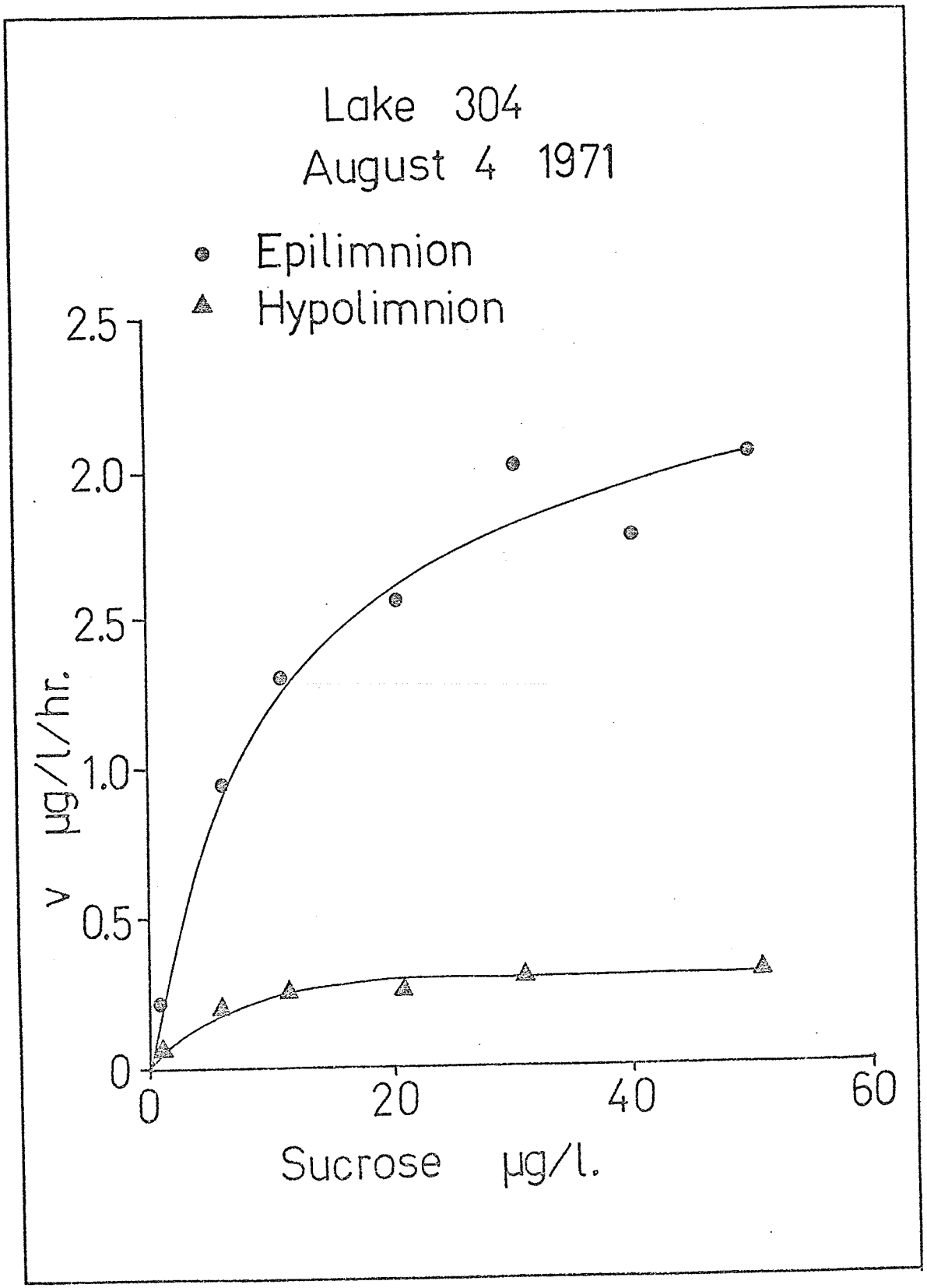

Lake 304
August 4 1971
Epilimnion
Hypolimnion
2.5
2.0
2.5
1.0
0.5
0
v µg/l/hr.
0
20
40
60
Sucrose µg/l.

Fig. 5. S/v against S, Lake 304, August 4, 1971.

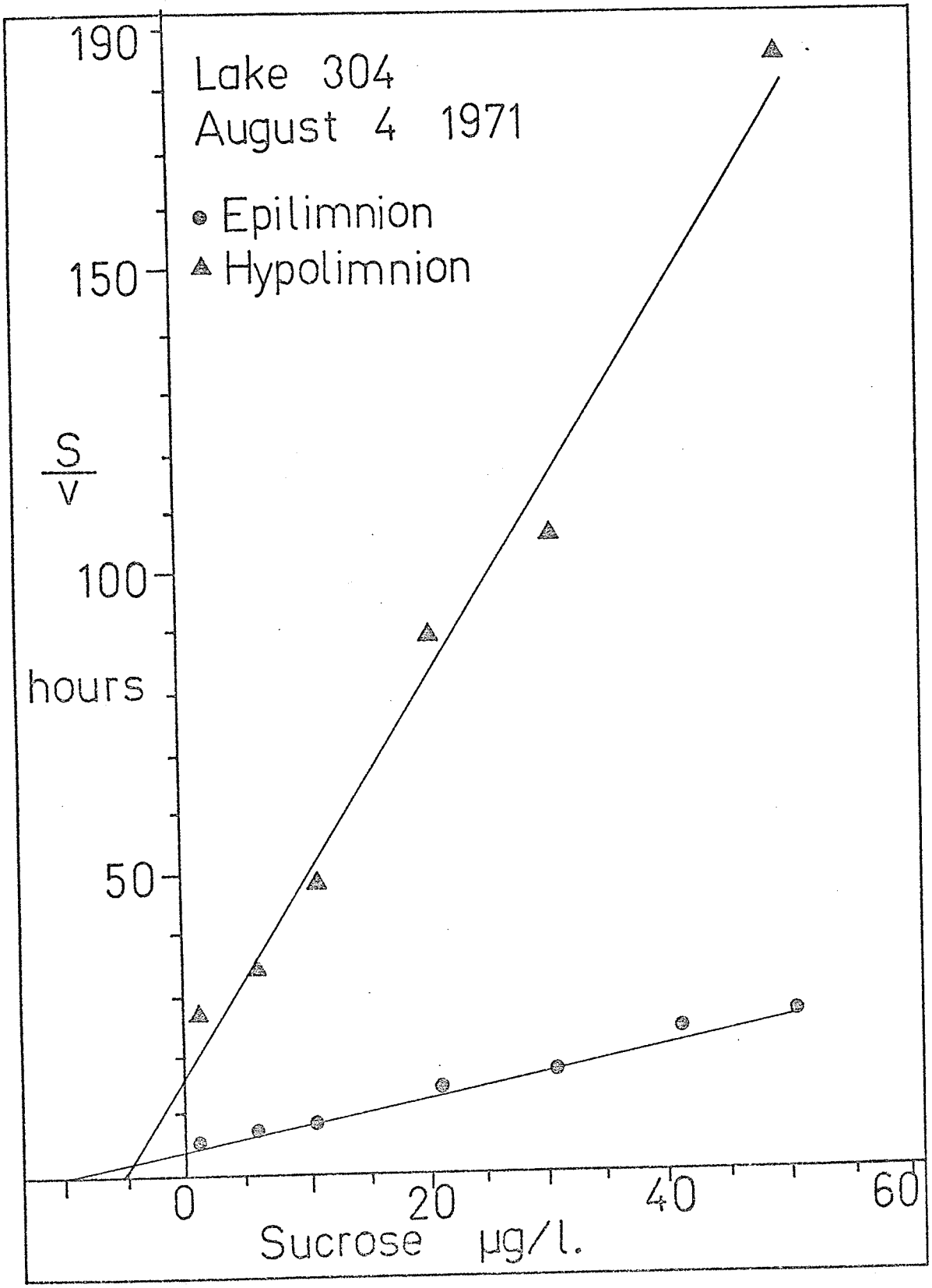

190
150
100
50
Lake 304
August 4 1971
Epilimnion
Hypolimnion
S/V
hours
0
20
40
60
Sucrose µg/l.

Fig. 6. V_{max}, Lake 304, July 1971 to January 1972.

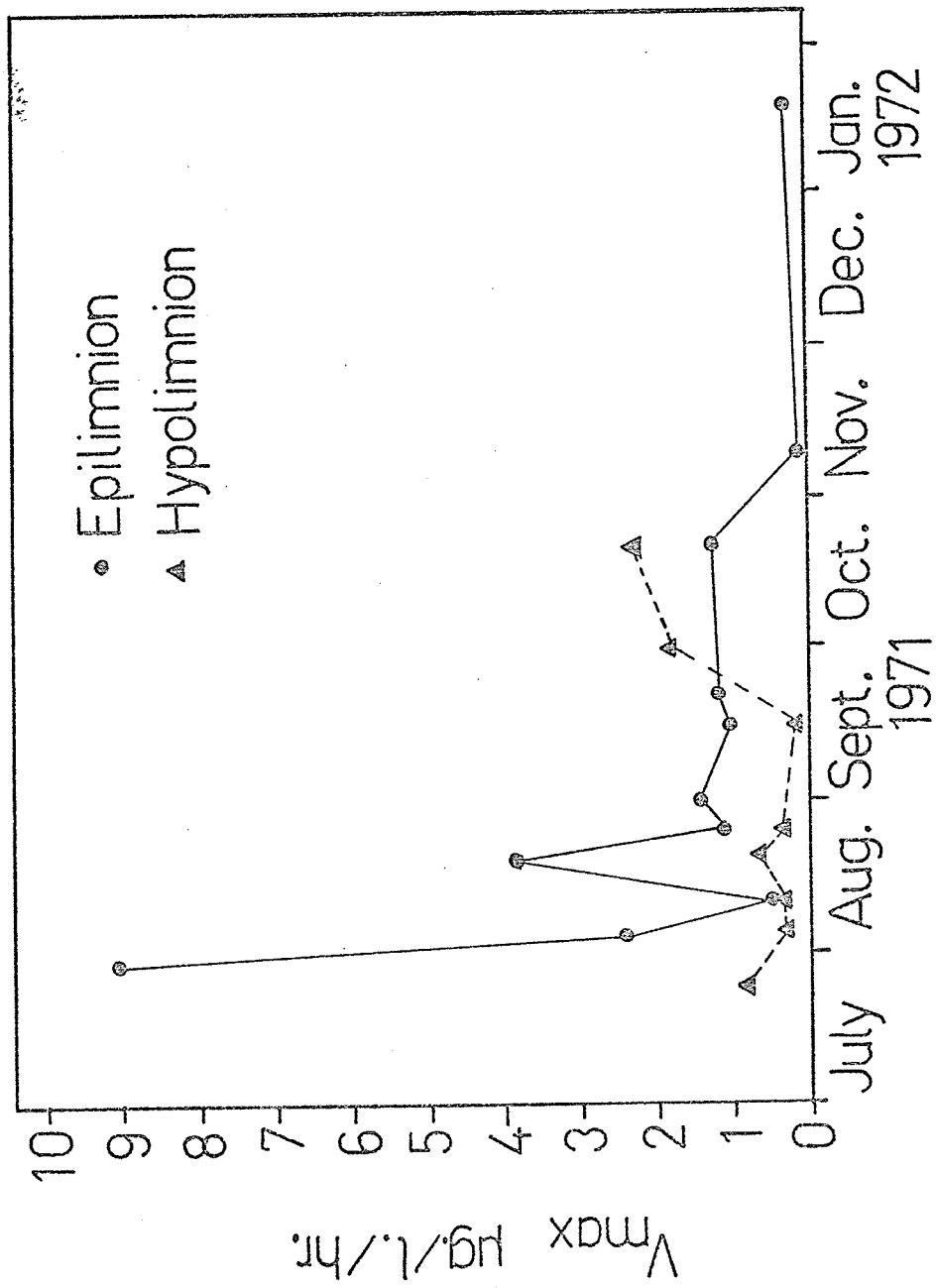

• Epilimnion
▲ Hypolimnion
V_{max} µg./L./hr.
0
1
2
3
4
5
6
7
8
9
10
July
Aug.
Sept.
Oct.
Nov.
Dec.
Jan.
1971
1972

Fig. 7. T_t, Lake 304, July 1971 to January 1972.

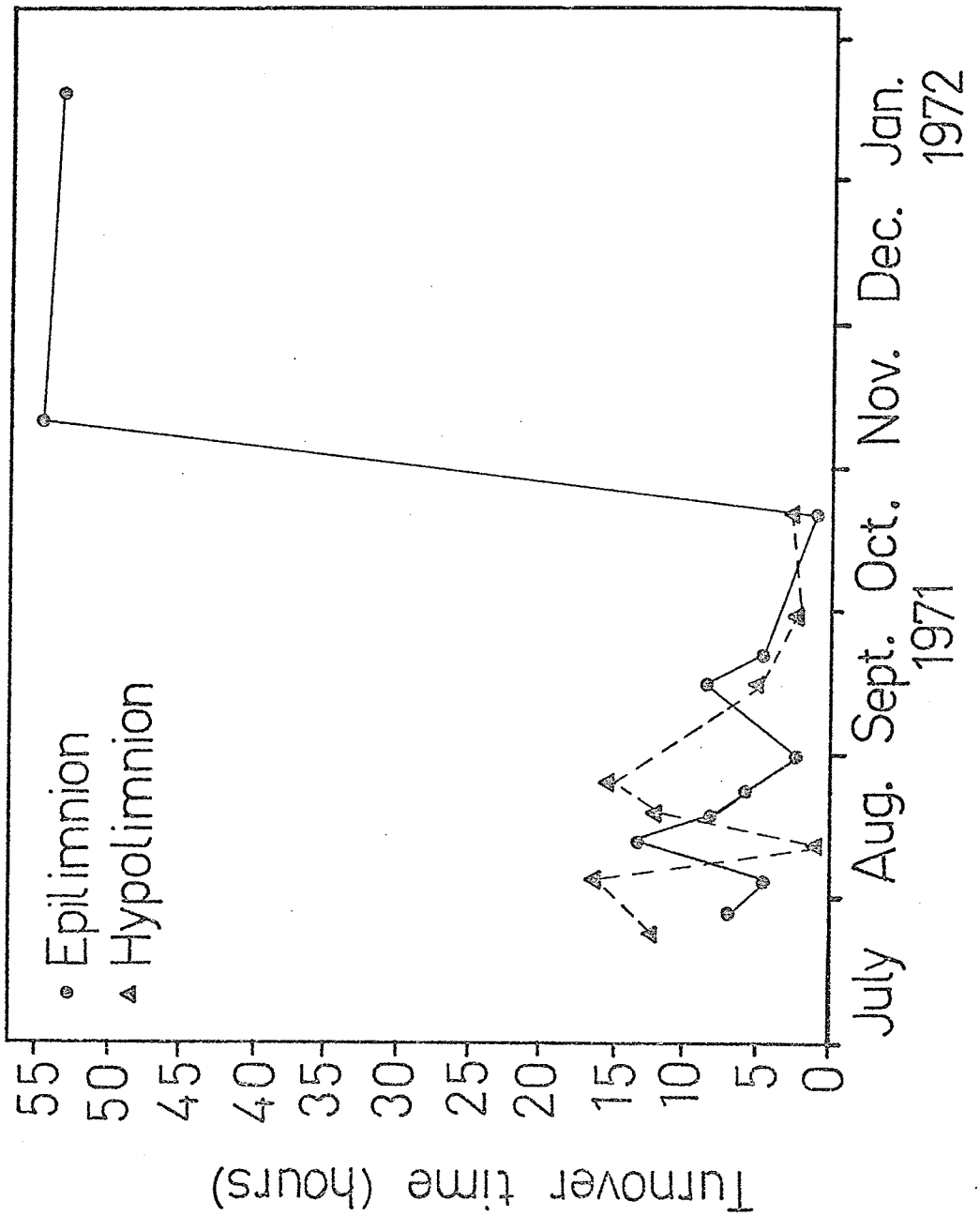
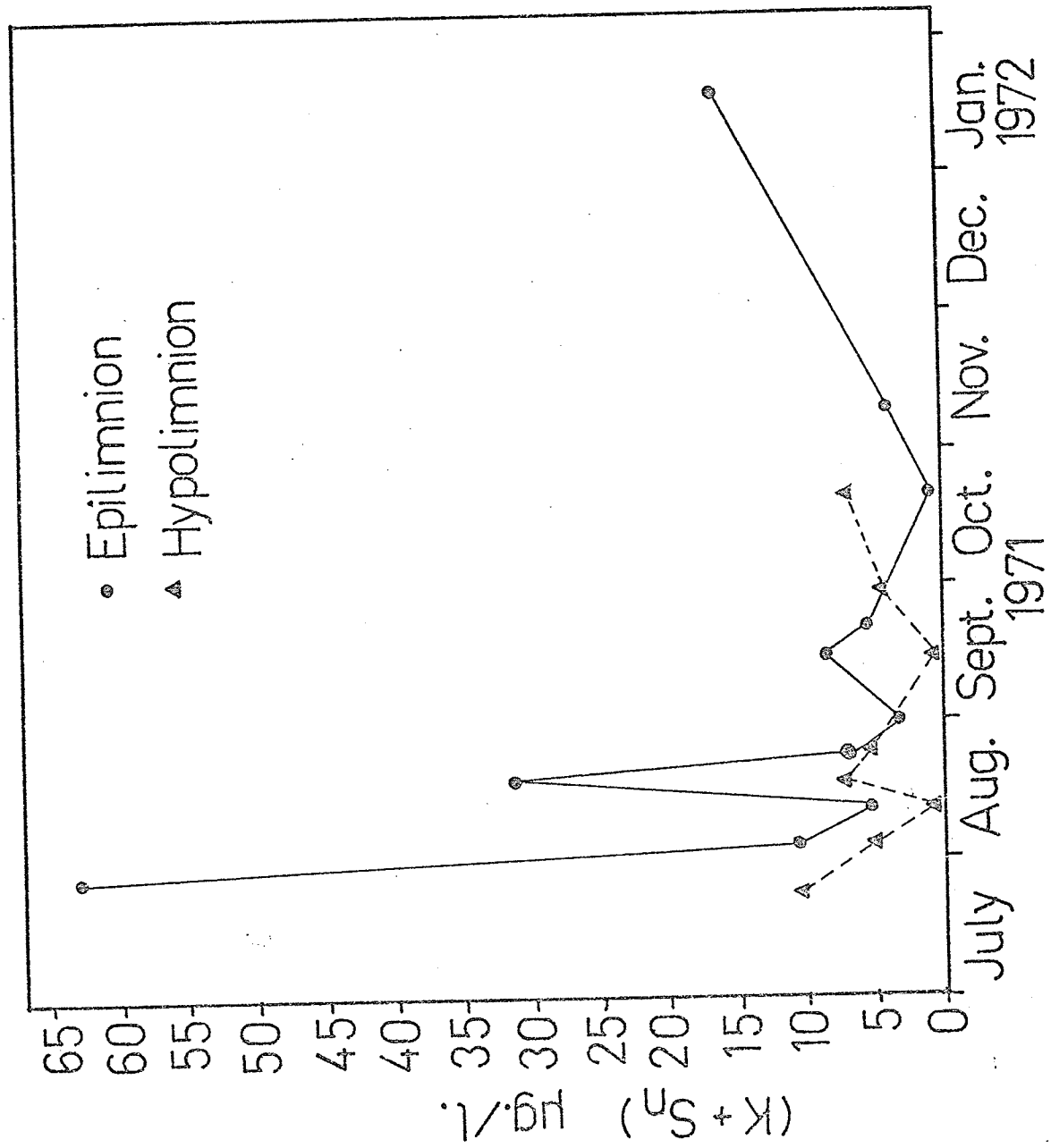

Turnover time (hours)
55
50
45
40
35
30
25
20
15
10
5
0
• Epilimnion
▲ Hypolimnion
July
Aug.
Sept.
Oct.
Nov.
Dec.
Jan.
1971
1972

Fig. 8. $(K + S_n)$, Lake 304, July 1971 to January 1972.


• Epilimnion
▲ Hypolimnion
(K + Sn) µg./l.
65
60
55
50
45
40
35
30
25
20
15
10
5
0
July
Aug.
Sept.
Oct.
Nov.
Dec.
Jan.
1971
1972

indicates that a larger number of micro-organisms capable of utilizing sucrose were present in the epilimnion than in the hypolimnion during summer. In the epilimnion V_{max} fluctuated between 9.1 and 0.46 µg/l/hr during July and early August, levelling off to around 1.2 µg/l/hr from late August through September and October. November and January values were low (0.07 and 0.31 µg/l/hr respectively). These low winter values may be due to low water temperatures (1-2°C), population decrease or decreased adaptation of micro-organisms following cessation of sucrose additions. Hypolimnetic V_{max} values ranged between 0.13 and 0.87 µg/l/hr until the end of September and October when V_{max} was 1.9 and 2.3 µg/l/hr respectively. These latter two values are higher than those during the summer and are possibly due to mixing of the water column during overturn which carried the denser epilimnetic microbial population to deeper waters combined with mixing of micro-organisms from the sediment.

It has been suggested (27) that V_{max} can be used as an index of eutrophication. It is interesting to note that values of V_{max} obtained for sucrose utilization in Lake 304 are of the same order of magnitude as the V_{max} obtained for glucose and acetate in two eutrophic Swedish lakes, Lakes Norrviken and Lötsjön (27).

The turnover time of sucrose remaining in Lake 304 just prior to enrichment was more often less in the epilimnion than in the hypolimnion until mid September. Epilimnetic values during this time period varied between 2.3 and 13 hours; hypolimnetic values varied between 0.66 and 16 hours. The former value was abnormally low and turnover time was more usually close to 14 hours. Turnover time, both in the epilimnion and hypolimnion, decreased from about 7.3 hours in mid September to

around 1.8 hours by the latter half of October. This decrease in the epilimnion is probably due to increased microbial activity resulting from the upwelling of nutrients from the bottom waters during fall overturn. During November and January the turnover time of sucrose in the epilimnion increased by an order of magnitude over the October value. This is to be expected owing to lowering of water temperature and corresponding reduction of microbial metabolic rates.

The $(K + S_n)$ of heterotrophic micro-organisms in the epilimnion was generally greater than that in the hypolimnion (Fig. 8). Since no measurement of S_n was made, the individual values of K and S_n are unknown. During July and August $(K + S_n)$ in the epilimnion fluctuated between 3.3 and 63 µg/l. From late August to early November $(K + S_n)$ lay between 0.99 and 8.7 µg/l; in January it rose to 16 µg/l. This latter figure probably represents a population unadapted to sucrose since sucrose additions had ceased three months previously. Values of $(K + S_n)$in the hypolimnion ranged between 0.26 and 11 µg/l with a mean of 5.2 µg/l.

D-glucose and D-fructose Utilization in Lake 304

Incorporation of sucrose, D-glucose and D-fructose by heterotrophic micro-organisms in the epilimnion of Lake 304 was measured on two occasions. Results are shown in Table 5. On both dates $(K + S_n)$ for glucose was three to four times greater than that for sucrose or fructose. Since V_{max} and T_t for glucose were also large, it is likely that a large population of micro-organisms poorly adapted to low concentrations of glucose were present. Fructose utilization exhibits a low V_{max} and high T_t in comparison with glucose and sucrose. This indicates a low

TABLE 5

Uptake of Sucrose, D-glucose and D-fructose by heterotrophic micro-organisms in the epilimnion of Lake 304.

Date	Substrate	$(K + S_n)$ ug/l	V_{max} ug/l/hr	T_t hours	Correlation %
31/8/71	Sucrose	3.3	1.4	2.3	94
	Glucose	13	1.9	7.0	96
	Fructose	4.9	0.62	8.0	92
15/9/71	Sucrose	8.7	1.0	8.6	91
	Glucose	28	1.9	15	94
	Fructose	8.3	0.55	15	89

population of micro-organisms able to utilize low levels of fructose. Sucrose uptake shows low $(K + S_n)$, moderate V_{max} and low T_t. This suggests the presence of a moderately large population of micro-organisms well adapted to the utilization of low concentrations of sucrose. In comparison, the turnover time of sucrose in an aquarium microcosm was found to be less than either of its constituents (48), though $(K + S_n)$ for sucrose was an order of magnitude greater than $(K + S_n)$ for glucose or fructose.

Sucrose utilization in the presence of D-glucose and D-fructose, Lake 304

Micro-organisms have been shown to possess phosphotransferase systems for the transport of carbohydrates (49). In an attempt to study possible interactions between substrates competing for the same site, uptake of sucrose by heterotrophic micro-organisms in the epilimnion of Lake 304 was measured in the presence of 5, 10 and 20 µg/l of both D-glucose and D-fructose. Results are shown in Table 6.

Fructose appears to have no significant effect upon sucrose uptake. Glucose appears to inhibit sucrose uptake noncompetetively; $(K + S_n)$ for sucrose increases from 4.3 at 5 µg glucose/l to 14 at 20 µg glucose/l indicating glucose to affect the affinity of the uptake system for sucrose. A possible mechanism of inhibition is combination of glucose with the sucrose uptake system at a site not identical to the sucrose-binding site. Competetive inhibition would not be expected since sucrose and glucose exhibit different molecular configurations.

It has been observed in an aquarium microcosm (48) that uptake of 1 µg/l of sucrose is inhibited by 1 mg/l glucose and fructose.

TABLE 6

Uptake of Sucrose by heterotrophic micro-organisms in the epilimnion of Lake 304 in the prescence of varying amounts of D-Glucose and D-Fructose, September 22, 1971.

Substrate	$(K + S_n)$ µg/l	V_{max} µg/l/hr	T_t hours	Correlation %
Sucrose	5.9	13.	4.6	94
Sucrose + 5 µg/l glucose	4.3	1.4	3.2	90
Sucrose + 10 µg/l glucose	7.6	1.3	6.1	93
Sucrose + 20 µg/l glucose	14	1.6	8.8	96
Sucrose + 5 µg/l fructose	5.8	1.7	3.5	97
Sucrose + 10 µg/l fructose	6.4	2.1	3.1	96
Sucrose + 20 µg/l fructose	7.4	1.8	4.2	99

Vallentyne and Whittaker (31), in a study of two lakes in eastern Ontario, found maximum glucose and sucrose concentrations of 5 and 10 μg/l respectively. Therefore it is unlikely that free sugars occur naturally in lake water at concentrations of 1 mg/l. Lake 304 receives 200 μg sucrose per liter per week. Even with no mixing between the epilimnion and the hypolimnion concentrations would not be expected to reach 1 mg/l except for short periods, in localized areas.

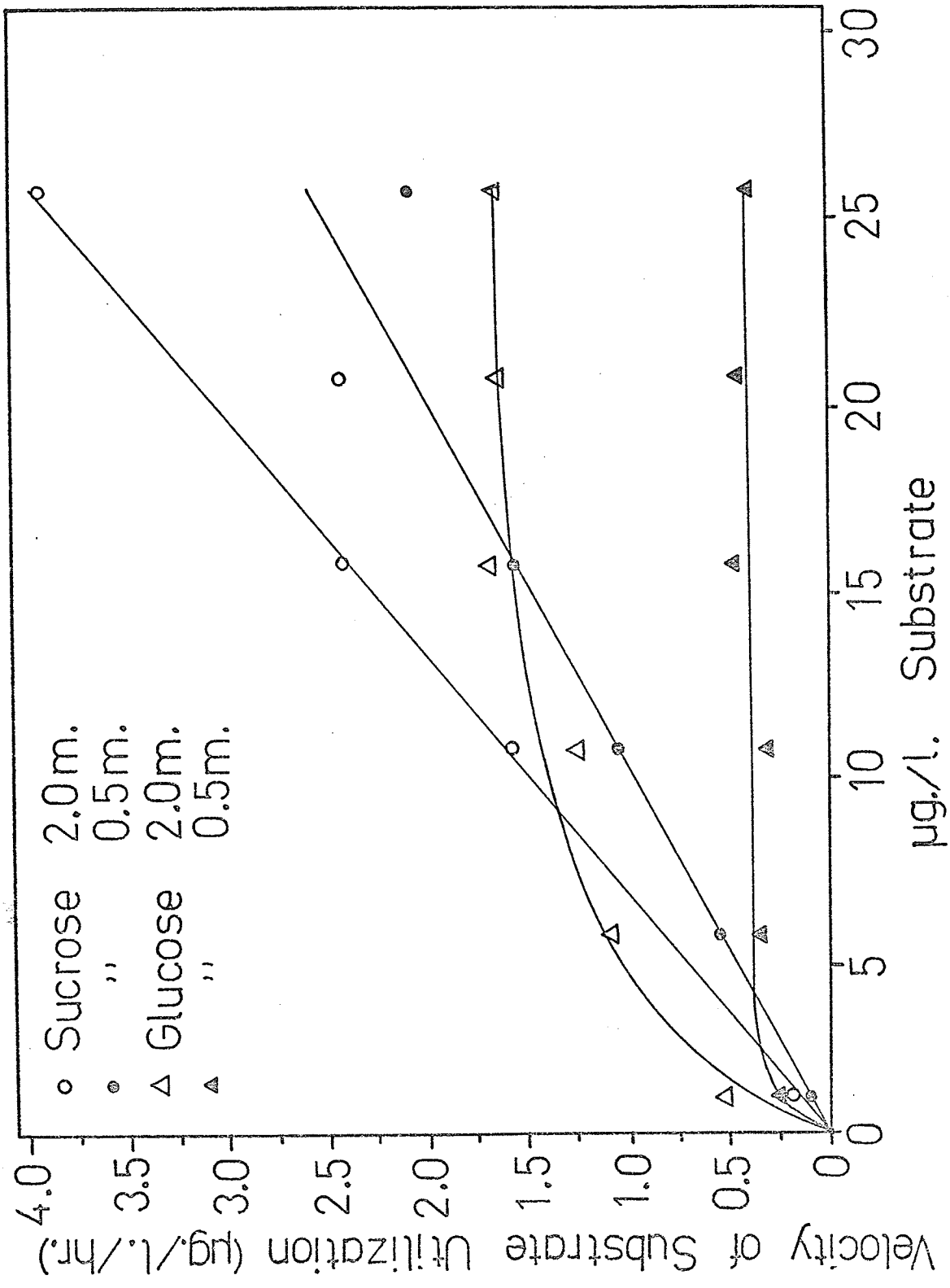
More recently Wood (50) has investigated the uptake by heterotrophic micro-organisms in an aquarium microcosm of three monosaccharides and four disaccharides with respect to inhibition by varying concentrations of each other. He concluded that a heterotrophic population possesses multiple substrate uptake systems. Carbohydrates were seen to affect each other's uptake in a manner which would suggest non-competitive inhibition. The utilization of a single carbohydrate is therefore partly dependent upon the concentration of other carbohydrates in the milieu.

Utilization of D-glucose and Sucrose in Lake 227

Utilization of glucose and sucrose at different depths in the epilimnion of Lake 227 was measured on two occasions. Figure 9 shows a velocity of utilization vs substrate concentration plot of the data obtained on July 26, 1972; data obtained on the other sampling date showed a similar pattern.

Utilization of glucose by heterotrophic micro-organisms in Lake 227 exhibits typical Michaelis-Menten uptake kinetics. Sucrose is

Fig. 9. Utilization of Sucrose and D-Glucose in Lake 227, July 26, 1972.


Velocity of Substrate Utilization (µg./l./hr.)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
5
10
15
20
25
30
µg./l. Substrate
Sucrose 2.0m.
" 0.5m.
Glucose 2.0m.
" 0.5m.

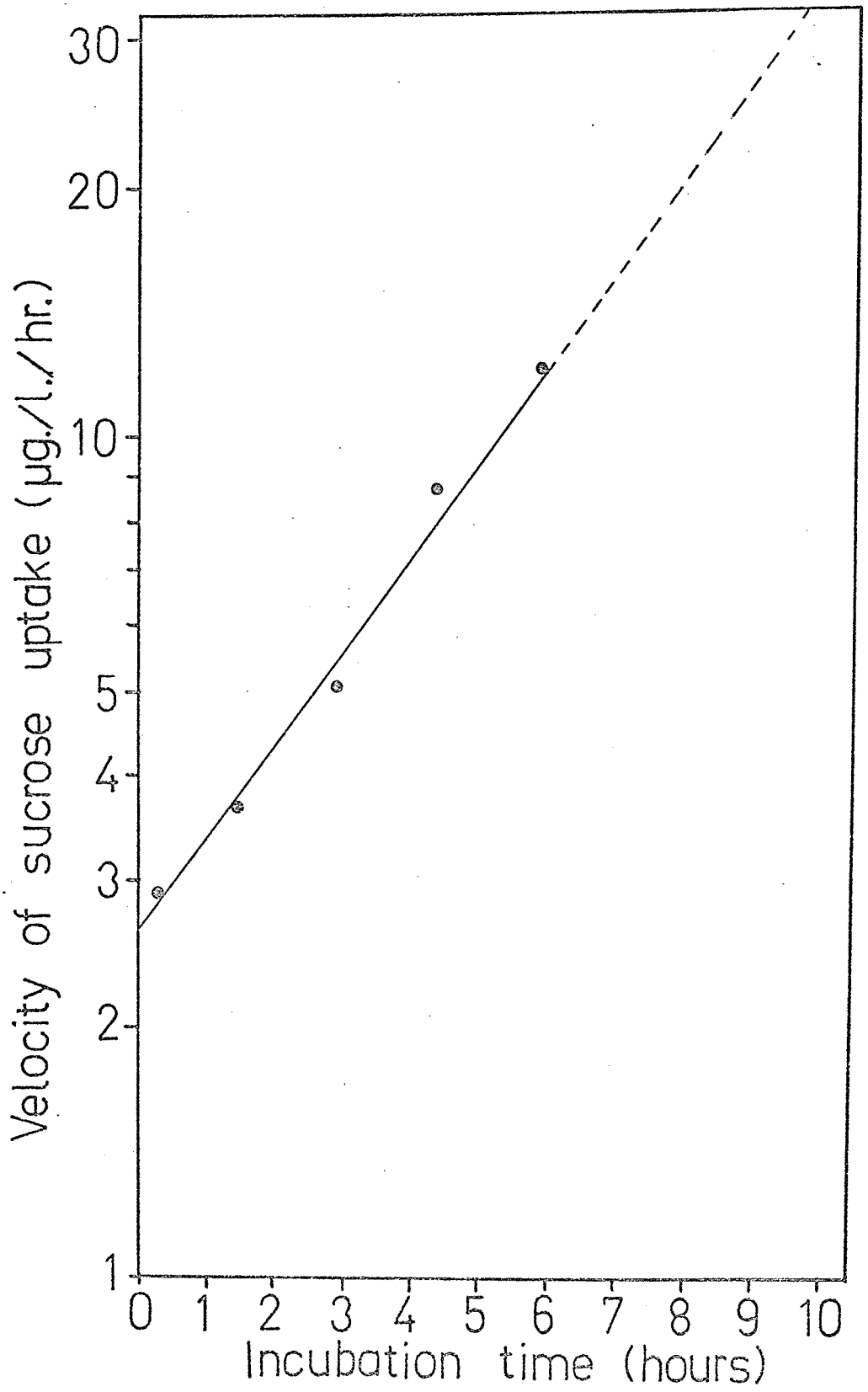
seen to diffuse into the micro-organisms by means of passive mechanisms. This demonstrates that the micro-organisms in Lake 227 are completely unadapted to utilizing sucrose as a nutrient and that sucrose is not a normal substrate in this lake. It is not known whether bacteria or algae are responsible for this passive uptake of sucrose at low levels.

Laboratory enrichment experiment

The change in velocity of sucrose uptake with time after addition of 200 µg/l sucrose was calculated as described in the Materials and Methods section (page 21). Up to six hours after enrichment, velocity increased exponentially (Fig. 10); after six hours, sampling times were too far apart to accurately calculate remaining substrate.

The exponential increase in velocity of uptake of sucrose may indicate exponential growth of heterotrophic micro-organisms. Assuming this trend to continue, and taking respiration into account, complete removal of sucrose would take 8 to 9 hours. Although a direct extrapolation from a carboy to a lake cannot be made, the laboratory experiment indicates that at this time of the year (September 8) enrichment leads to a rapid increase in microbial numbers. This large population is capable of reducing sucrose concentrations to low levels within about a day. These data cannot be extrapolated to early summer however as the microbial population may have been small or unadapted due to the schedule of sucrose additions.

Fig. 10. Velocity of sucrose uptake with time after addition of 200 μg sucrose per liter of Lake 304 epilimnetic water, September 8, 1971.


Velocity of sucrose uptake (µg./l./hr.)
30
20
10
5
4
3
2
1
0 1 2 3 4 5 6 7 8 9 10
Incubation time (hours)

Uptake of Sucrose pre and post enrichment

On two occasions heterotrophic uptake of sucrose in Lake 304 was studied immediately before and after the weekly enrichment. Results obtained on August 17 and 18, 1971 are shown in Table 7. It is clear from these results that conditions below the thermocline remain unchanged immediately after enrichment. In the epilimnion, the turnover time of sucrose in the water increases from 8.1 hours to 130 hours after enrichment. V_{max} decreases by an order of magnitude after enrichment, and $(K + S_n)$ remains approximately the same. This outcome is somewhat contradictory; since sucrose has been added to the lake, it would be expected that $(K + S_n)$ would increase considerably after enrichment.

Several explanations of this phenomenon can be advanced. Firstly, the method of enrichment employed has been shown to lead to temporary patchy distribution of nutrient (51). Enrichment of Lake 304 proceeds by mixing nutrient with water let into the bottom of a boat and allowing the dissolved nutrient to be drawn out of the drainage hole of the boat by Venturi effect while the boat is driven around the lake. Enrichment in the immediate vicinity of the sampling station is generally avoided so as not to disturb the primary production bottles suspended from the buoy. An analysis of nitrate distribution shortly after enrichment in Lake 227 revealed 600 µg N/l at one point in the epilimnion (51), whereas even mixing over the upper 3 m of the lake would produce a concentration of about 110 µg N/l. Therefore sucrose concentration at the sampling station may not be greatly increased immediately after enrichment. The reduction of V_{max} occurred too quickly to be accounted for by zooplankton predation.

TABLE 7

Uptake of Sucrose in Lake 304, pre and post enrichment, August 17 and 18, 1972.

		$(K + S_n)$ µg/l	V_{max} µg/l/hr	T_t hours	Correlation %
Epilimnion	pre enrichment	31	3.9	8.1	98.49
	post enrichment	40	0.31	130	94.15
Hypolimnion	pre enrichment	7.4	0.61	12	95.66
	post enrichment	3.6	0.32	11	95.14

Secondly, there is some evidence of diffusion occurring at higher substrate concentrations. Considering only the data points obtained at the three lower substrate concentrations, an S/v against S plot generates the following kinetic parameters: $(K + S_n)$ of 6.8 µg/l, V_{max} of 0.072 µg/l/hr and T_t of 97 hours (correlation 97%). It must be noted that three data points are unsatisfactory from a statistical point of view, and the value for V_{max} thus obtained is abnormally low. A similar plot of the four data points obtained at higher substrate concentration generates a curve of gentler gradient. In this case $(K + S_n)$ is 520 µg/l, V_{max} 2.3 µg/l/hr and T_t 220 hours. However, data points are quite widely scattered around this line.

Thirdly, the concomitant addition of nitrogen and phosphorus may temporarily effect the physiology and metabolism of heterotrophic micro-organisms. The extent or nature of this phenomenon is unknown.

The second post-enrichment measurement of heterotrophic uptake of sucrose yielded results which were not statistically reliable enough to provide any firm conclusions. However, results at 1.5 m post enrichment indicated a slight increase in $(K + S_n)$ compared with epilimnetic values of the day before (7.1 to 24 µg/l). V_{max} decreased by almost an order of magnitude, and T_t increased from 5.9 to 240 hours, similar to the previous experiment.

These experiments indicate that enrichment influences the population of heterotrophic micro-organisms in the epilimnion, although the reason for the large post enrichment decrease in V_{max} is not clear.

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Several questions may be asked concerning the fate of the sucrose added to Lake 304. Firstly, how long does it take for the micro-organisms in Lake 304 to become adapted to weekly addition of sucrose? A consideration of changes in V_{max} (Fig. 6, p. 35) indicates that microbial activity fluctuates rather markedly during late July and most of August. This fluctuation represents the damped oscillations of a perturbed system, reaching equilibrium in late August. The steady state which existed prior to enrichment was disturbed. Time was required for the development of a population of micro-organisms capable of effectively utilizing sucrose, since sucrose was not a normal substrate (p. 48). Changes in the population of zooplankton capable of grazing the micro-organisms would also be necessary before a new steady state could be reached. Data concerning the utilization of sucrose in Lake 304 during May and June 1971 were not available. However, data for the rest of the summer indicates that the micro-organisms reached a stable state with respect to sucrose utilization about three months after the commencement of enrichment.

Secondly, one may ask where the added sucrose is situated in the water column. It has been observed in Lake 227 that nitrate added as $NaNO_3$ enters particulate matter and remains in the epilimnion (51). Similarly, no increase in sodium is observed below the thermocline; sodium appears in the lower depths of the lake only after fall overturn. The technique of enrichment is identical in Lake 304, so it would be expected that sucrose remains above the thermocline and is utilized there. This conclusion is also supported by the greater activity with

respect to sucrose of micro-organisms in the epilimnion. It is possible that the sucrose may be localized in a layer within the epilimnion; however, no firm evidence for this is available. The presence of heterotrophic micro-organisms adapted to sucrose below the thermocline introduces the possibility of sucrose reaching the hypolimnion. It is believed that these micro-organisms are carried from the epilimnion by zooplankton or descend through the thermocline by sedimentation. Definite conclusions concerning the location of sucrose in the water column cannot be drawn without knowledge of the concentration of sucrose in the water. It is expected that this information will become available in the near future.

Thirdly, how fast is the added sucrose utilized by heterotrophic micro-organisms? The laboratory enrichment experiment of September 8 (p. 48) indicates that 200 µg sucrose/l can be utilized within 9 hours of enrichment under conditions of complete mixing. Since the added sucrose remains in the epilimnion, 266 µg sucrose/l would be present when the thermocline began at 3 m, assuming even mixing throughout the epilimnion. It is probable that towards the end of the summer when the heterotrophic micro-organisms were adapted to sucrose as a nutrient, a rapid increase in microbial numbers and hence microbial activity was capable of reducing the weekly addition of sucrose to a low level within a day. Grazing by zooplankton could effectively reduce microbial numbers by the next sampling date thus reducing microbial activity.

Fourthly, what is the relation of heterotrophic utilization of sucrose to primary productivity? If the weekly addition of sucrose were evenly distributed through the upper 3 m of Lake 304, a concentration

of 266 µg per liter would be obtained. Studies of the mineralization of sucrose by heterotrophic micro-organisms in Lake 304 have shown that 25 to 35% of total sucrose utilized is available as free CO_2. This is an underestimate of CO_2 available for incorporation into biomass, since some has undoubtedly been assimilated through bacterial metabolism during incubation. However, taking 30% as a conservative estimate of CO_2 available, 2.8 µM CO_2/l/week could be generated from 266 µg sucrose/l/week. The lowest levels of CO_2 in the epilimnion of Lake 304 during summer 1971 was around 6 µM CO_2/l, values generally varying between 10 and 20 µM CO_2/l (51). These measurements were made at about 10 a.m. The concentration of CO_2 in the epilimnion of Lake 227 has been shown to vary diurnally and is highest just before dawn (52). If this is also true for Lake 304, it may be assumed that 2.8 µM CO_2/l/week is a low but not insignificant fraction of the total CO_2 available for primary productivity. Data pertaining to the cycling of sucrose-carbon through bacteria, algae and zooplankton is not available.

It is possible to compare pre-enrichment daily rates of primary productivity (calculated from the rate between 10 a.m. and 2 p.m.) with the rate at which sucrose is converted into particulate carbon by heterotrophic processes and the rate of total sucrose-carbon utilization. The kinetic parameters for sucrose uptake were corrected for filtration error and mineralization of sucrose. S_n was taken as $(K + S_n)$ which is an overestimation. The velocity of uptake and utilization at a sucrose concentration of S_n was calculated and converted to mg C/m^2/day down to 3 m, and compared with primary production down to this depth.

This treatment of the data showed that heterotrophic conversion of sucrose to particulate carbon from July to October 1971 inclusive is, on average, 8.24% of primary production in the epilimnion. The range was 41.48% to 0.85%. The former figure was obtained during July when the micro-organisms were not completely adapted to sucrose, hence S_n was larger than normal. The latter figure was obtained during the fall phytoplankton bloom when primary production was higher than normal. Generally, values of sucrose-carbon converted to particulate material varied between 2% and 17% of carbon fixed by photosynthesis in the epilimnion.

Considering total sucrose-carbon utilization by heterotrophic micro-organisms in the epilimnion, this represented a mean of 12% of carbon fixed by primary production, all but two of the values lying between 4 and 27%. However, if there is a burst of algal carbon fixation shortly after dawn similar to that observed in Lake 227 (52), comparison of heterotrophic carbon fixation with algal carbon fixation during the middle of the day leads to an overestimation of the role of heterotrophic microorganisms.

During the period of enrichment (May 26 to October 13, 1971) a net gain in suspended carbon of 8.3 g C/m^2 was observed. However, during the period in which heterotrophic utilization of sucrose in Lake 304 underwent intensive study (July 28 to October 20, 1971) there was a loss of 8.84 g suspended carbon per m^2. This loss of carbon may be a result of the sedimentation of particulate matter or loss of carbon dioxide to the atmosphere. The importance of these processes in removing carbon from active cycling among the aquatic communities of Lake 304 is not known.

An apparent result of the addition of sucrose to Lake 304 was the lowering of pH in the epilimnion from 6-7 to 5-6. In contrast, the pH of the epilimnion of Lake 227, enriched with N and P only, increased to around 10. This difference was not a result of adding N as NH_4Cl to Lake 304, since during 1972 N was added to Lake 304 as $NaNO_3$ (as in the case of Lake 227) and the pH remained low. The mechanisms producing this phenomenon are not known.

It is important to note that although C, N and P were added to Lake 304, this lake did not become as highly eutrophied as Lake 227 which received only N and P. Phytoplankton standing crop (as chlorophyll a) in Lake 227 increased from 1-5 µg/l to 100-300 µg/l after enrichment, while that in the epilimnion of Lake 304 rarely exceeded 50 µg/l. It has been shown (52) that the phytoplankton in Lake 227 can obtain much of the carbon for primary production from atmospheric carbon dioxide. Although the sucrose added to Lake 304 can be effectively utilized by heterotrophic micro-organisms and a portion of the carbon made available to the phytoplankton for primary production, an allochthonous source of organic carbon is in this case not a prerequisite to the development of a highly eutrophied state in Lake 304.

At the present time, the techniques used in this study are the most useful available for analysis of the role of heterotrophic micro-organisms in aquatic environments. However, the necessity of simultaneously measuring incorporation and mineralization of substrate as well as obtaining at several substrate concentrations the error due to filtration, renders the technique somewhat unwieldy. It has been suggested (53) that it may be possible to develop a technique analagous to that of Schindler (54) for primary production, which would eliminate the need to filter samples.

APPENDIX I

Mineralization of Sucrose

Introduction

Sucrose entering the heterotrophic cell may be assimilated into cellular material, excreted as soluble organics, or used to obtain energy through respiration with accompanying release of carbon dioxide. Assimilation is measured by counting radioactivity in particulate material. Collection of $^{14}CO_2$ enables a better estimation of mineralized substrate, but excretion must be assumed to be negligible.

Several methods have been devised to collect $^{14}CO_2$. Hobbie, Crawford and Webb (24) used an ion chamber to collect $^{14}CO_2$ which was subsequently counted with an electrometer. Williams and Askew (7) flushed out $^{14}CO_2$ with a stream of CO_2-free air and converted it to $Ba^{14}CO_3$ which was counted. Hobbie and Crawford (8) devised a simpler method in which a water sample incubated in an Erlenmeyer flask sealed with a serum stopper was acidified at the end of the incubation period; $^{14}CO_2$ was collected in phenethylamine soaked on a filter paper wick suspended in the flask in a rod-and-cup assembly. This technique was slightly modified by Harrison, Wright and Morita (9) who substituted serum bottles for Erlenmeyer flasks. This latter method was compared with a closed-loop system incorporating a push-pull diaphragm pump similar to those used to aerate aquaria (55).

Materials and Methods

0.1 μCi $NaH^{14}CO_3$ was added to eight 100 ml serum bottles containing 50 ml distilled water. Four of the bottles were sealed with conventional serum stoppers with a rod-and-cup assembly equivalent to

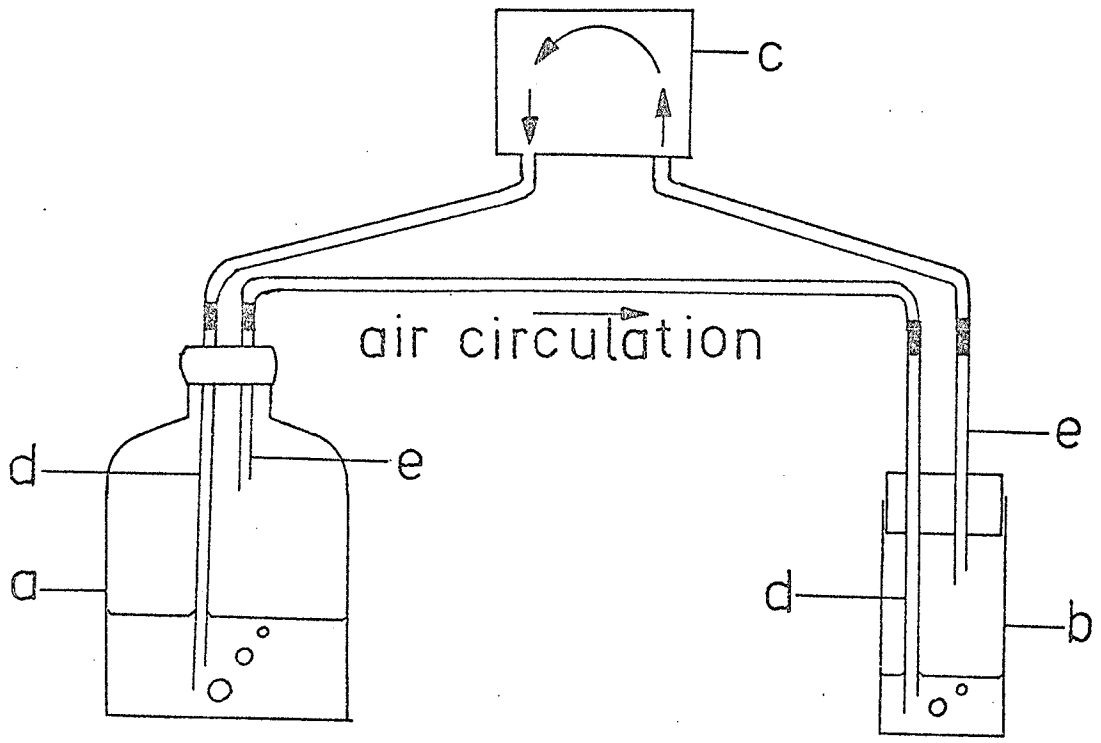
that of Hobbie and Crawford (8) passing through the septum. However, an accordian-folded 20 x 40 mm piece of Whatman GF/C filter paper was used as a wick since it was found that no quenching correction was necessary when using GF/C in place of Whatman No. 1. The other four bottles were sealed with normal serum stoppers, as were two bottles containing 50 ml distilled water and 0.1 μCi ^{14}C-sucrose (U).

$^{14}CO_2$ was driven out of solution by injecting 0.25 ml 2N H_2SO_4 through the septum of each serum bottle, which reduced the pH to around 2. It had previously been determined that sulfuric acid gives a higher percentage recovery of $^{14}CO_2$ than formalin. Hydrochloric acid is not suitable as it is volatile and degrades the phenethylamine.

Phenethylamine (Packard, Scintillation grade) (0.15 ml) was injected through the septum onto the folded GF/C. (A yellow discoloration producing color quenching sometimes occurs in phenethylamine and can be removed by distillation). These four bottles were placed on a rotating shaker (New Brunswick Scientific laboratory rotator model G2) at 250 rpm for varying time periods (10 min., 35 min., 1 hr. and 1.5 hr.) at the end of which the filter was placed in a glass scintillation vial containing about 15 ml of Woeller's fluor B (56). The glass vial was rinsed several times with this fluor and the rinsings added to the scintillation vial.

The apparatus used for collection of $^{14}CO_2$ from the remaining six bottles consisted of a closed loop system linking a sealed push-pull diaphragm pump (Universal Electric Co., Michigan, U.S.A.), one serum bottle and a glass scintillation vial by means of small-bore silicone rubber tubing (Fig. 11). Carbon dioxide losses are encountered if

Fig. 11. $^{14}CO_2$ collection apparatus.



a Serum bottle
b Scintillation vial
c Diaphragm pump
d 4 inch cannula
e 1.5 inch disposable hypodermic needle

plastic or rubber tubing is used. Incorporated into the silicone rubber tubing were two air inlets (4 inch sharpened cannulas) and two air outlets (1.5 inch 18 g disposable hypodermic needles). A cut-down silicone rubber stopper containing a needle-cannula pair was firmly inserted into the scintillation vial which contained 5 ml of Woeller's fluor A (56). At least 4 ml of Woeller's fluor A was required to absorb all the water vapour bubbled over. The other needle-cannula pair was inserted through the stopper of the serum bottle. Air circulation was initiated and maintained for a different time period for each of the four $NaH^{14}CO_3$-containing serum bottles (2.5, 5, 10 and 15 mins.). The two serum bottles containing ^{14}C-sucrose (U) were bubbled for five minutes each. After bubbling $^{14}CO_2$ from each bottle, the scintillation vial was removed and 10 ml of Woeller's fluor B added. Only 35% of total available volatile carbon is collected by this method when the sample is not acidified.

Samples were counted on a Picker Liquimat 220 liquid scintillation counter, together with four vials containing 0.1 ml ^{14}C-sucrose (U) and four vials containing 0.1 ml $NaH^{14}CO_3$ in Woeller's fluor B. Conversion of counts per minute to disintegrations per minute was accomplished by the channels ratio method utilizing an internal standard.

Results and Discussion

As shown in Table 8, $^{14}CO_2$ collection by circulation of air is more efficient and faster than by absorption onto a phenethylamine-soaked wick. Sucrose breakdown by acid and ^{14}C transferred in water vapour are negligible.

TABLE 8

Comparison of two methods for $^{14}CO_2$ collection.

Radiochemical	Collection method	Collection time	% label recovered
$NaH^{14}CO_3$	Phenethylamine-soaked	10 min.	24.55
	GF/C wick	35 min.	53.06
		1 hr.	78.86
		1.5 hr.	86.54
$NaH^{14}CO_3$	Bubbled through	2.5 min.	80.21
	phenethylamine	5 min.	96.66
		10 min.	97.23
		15 min.	94.70
^{14}C-sucrose(U)	Bubbled through	5 min.	0.42
	phenethylamine	5 min.	0.84

It has been reported (57) that the counting rate from phenethylamine ^{14}C-carbamate decreases with time owing to loss of $^{14}CO_2$, losses of up to 50% in three hours being observed. This phenomenon may be due to the lack of methanol in the fluor; methanol is necessary to increase the solubility of the carbamate and lower the partial vapour pressure of the amine. In this laboratory, no such loss was apparent in samples counted at intervals over six hours, and again after 24 hours.

Subsequent analyses of sucrose mineralization by naturally-occurring heterotrophic micro-organisms were performed using the closed-loop system. Each sample was bubbled for five minutes, and two or three acidified water samples containing $NaH^{14}CO_3$ were used to determine percentage recapture in each experiment.

It was not possible to estimate incorporation and mineralization of substrate from the same incubated sample. Total dpm in $^{14}CO_2$ and particulate material from the same sample were less than dpm in particulate material alone from a similar sample which was not acidified and bubbled. This indicates severe cell breakage and loss of non-volatile contents to the aquaeous phase during the $^{14}CO_2$ collection procedure. The contribution of acid-volatile ^{14}C-labelled compounds to $^{14}CO_2$ collected is not known.

APPENDIX II

Filtration Error

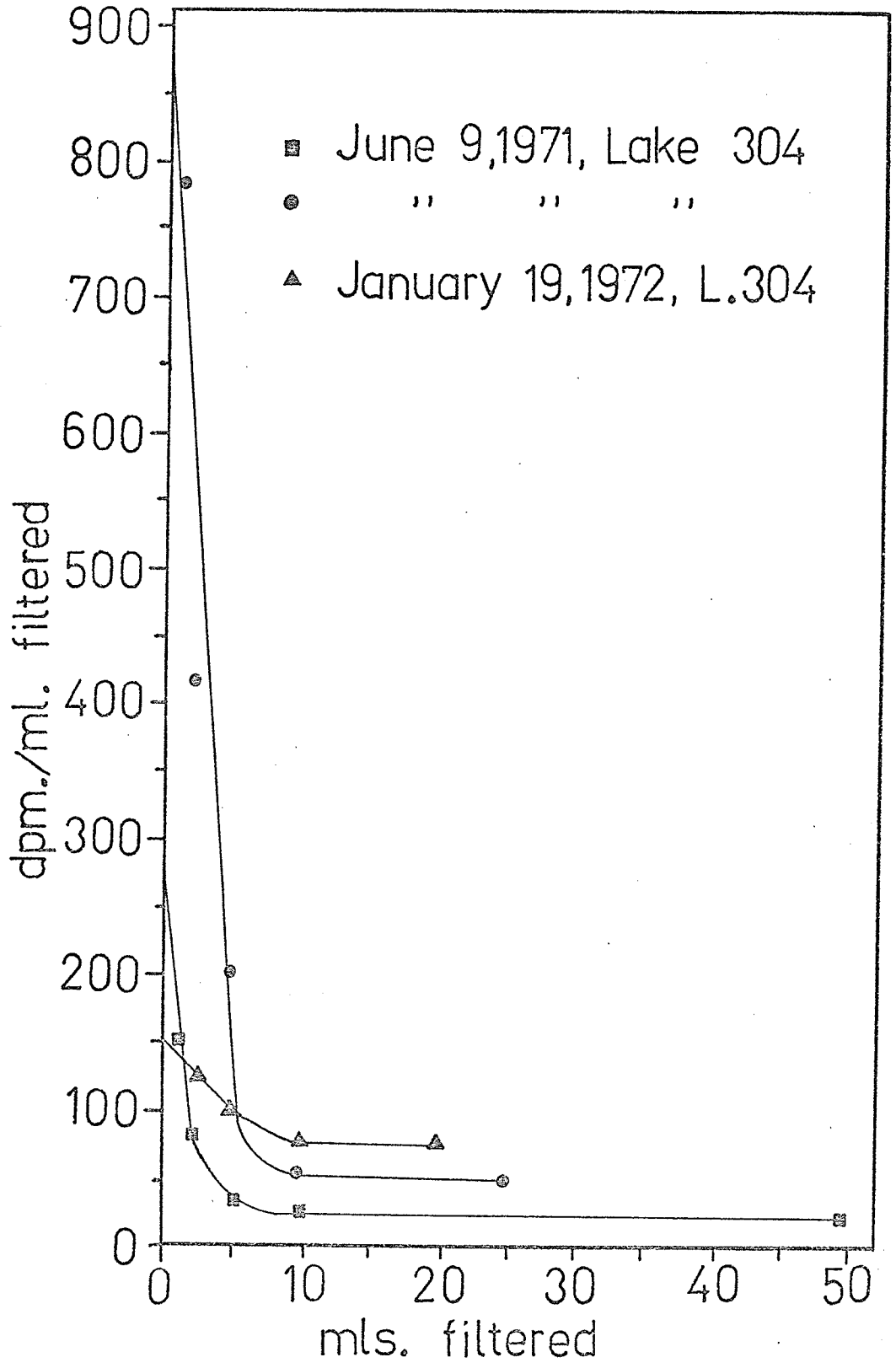
FILTRATION ERROR

Primary productivity data obtained by the ^{14}C method of Steemann-Nielsen (13) is subject to an error due to decrease in dpm per ml with increasing volume filtered. Arthur and Rigler (58) measured primary production by $NaH^{14}CO_3$ uptake and O_2 production, and found that up to half of the fixed carbon as measured by ^{14}C incorporation was lost during manipulation of incubated samples. ^{14}C results corrected for filtration error gave estimates of carbon fixation approximately the same as those obtained by the oxygen method. Cell breakage resulting in loss of ^{14}C-labelled contents on vacuum filtration was advanced as the cause of the filtration error.

The possibility of this error being inherent in the techniques used to study heterotrophic uptake was investigated. A filtration error was obtained on all but one of the occasions it was sought for; examples are shown in Fig.12. Correction factors from 1.1 to 6.9 times the dpm contained in a 5 ml aliquot obtained in this study are comparable to values ranging from 1.2 to 6.1 found by Schindler and Holmgren (59) during primary productivity studies on Shield lakes in the same area.

Berman and Williams (60) suggested that the part of a Millipore filter which lies between the solid flanges of a glass Millipore unit can absorb radioactive solution and cannot be effectively washed. This could lead to a filtration error since the volume of liquid absorbed around the periphery of the filter would remain constant regardless of volume filtered; dpm around the edge would therefore increase in proportion to total dpm when filtering decreasing volumes.

Fig. 12. Typical filtration correction curves obtained from Lake 304 using ^{14}C-sucrose(U) as a substrate.


900
800
700
600
500
400
300
200
100
0
dpm./ml. filtered
June 9,1971, Lake 304
" " "
January 19,1972, L.304
0
10
20
30
40
50
mls. filtered

Experiments to test this theory were performed on Lake 304 epilimnetic water incubated for 3 hours with 0.1 μCi ^{14}C-sucrose(U) per 50 ml and 30 or 40 μg. unlabelled sucrose per liter. Filtration was performed at a vacuum of less than 300 mm Hg.

Firstly, different volumes (20, 10, 5, 2 ml) were filtered through 47 mm 0.45 μ Millipore HA filters by pouring the water sample down the "candle" of a 25 mm Millipore filter unit standing inside the 47 mm unit. After taking the filter just to dryness, the candle was lifted from the filter surface and rinsed onto the filter with 5 ml of distilled water. The candle was then fully removed and the filter rinsed with a further 5 ml of distilled water, taking the filter just to dryness each time. The filter was dissolved in dioxane-based fluor (46) and counted. No significant differences was found between experimental and control results (filtered in normal manner) (Table 9).

Secondly, different volumes (50, 25, 15, 5, 2, and 1 ml) were filtered through 47 mm 0.45 μ Millipore HA filters. That part of the filter which lay between the glass flanges of the filter holder was cut off and counted separately from the centre of the filter. Results (Table 10) show that dpm around the periphery increased with increasing volume filtered, though dpm/ml decreased; dpm around the periphery as a percentage of total dpm increased as volume filtered decreased. This "edge" effect may be significant when measuring very low levels of heterotrophic uptake or primary production; it is not sufficient to account for the error observed during measurements of heterotrophic utilization of sucrose in Lake 304.

TABLE 9

Filtration error: Water sample filtered through the "candle" of a 25 mm Millipore filter unit.

Unlabelled Sucrose µg/l	ml filtered	dpm/ml	
		25 mm candle	Control
30	20	619	779
	10	857	870
	5	888	931
	2	1140	946
40	20	760	654
	10	959	907
	5	1001	1280
	2	1242	1256

TABLE 10

Filtration error: dpm per ml on center and on periphery of filter

ml filtered	dpm/ml		dpm on periphery as % of total dpm
	center	periphery	
50	1665	55	3.2
25	1993	85	4.1
15	2591	50	1.9
5	2452	203	7.6
2	2629	256	8.9
1	2568	319	11.1

Thirdly, hydrophobic edge 0.45 μ Millipore Filters (Millipore HAEP047AO) were used in the filtration of different volumes (50, 25, 10, 5, 2 and 1 ml) of water sample. Comparison with a control filtered through 0.45 μ Millipore HA filter (Table 11) shows no reduction in filtration error with the use of hydrophobic edge filters.

Absorption and adsorption of ^{14}C-sucrose(U) to the filter was found to be negligible. 100 ml aliquots of water from 2 m in Lake 240 were dispensed into acid-washed glass bottles and different concentrations of ^{14}C-sucrose(U) added (0.1, 0.3, 0.5 and 0.7 μCi per 100 ml.) The entire contents of each bottle was filtered through a 0.45 μ Millipore HA filter and rinsed twice with 5 ml distilled water. Between 0.03 and 0.06% of added label was retained on the filter.

The above experiments indicate that neither adherence of unincorporated radioactivity nor absorption of label around the periphery of the filter are responsible for the observed filtration error. Observed dpm in filtrate plus dpm on filter remains constant for different volumes filtered when a filtration error is present indicating that $^{14}CO_2$ loss is not the main factor involved (51). Primary productivity samples treated with acid and bubbled to remove inorganic ^{14}C (54) generate results identical to those obtained from filtered samples corrected for filtration error.

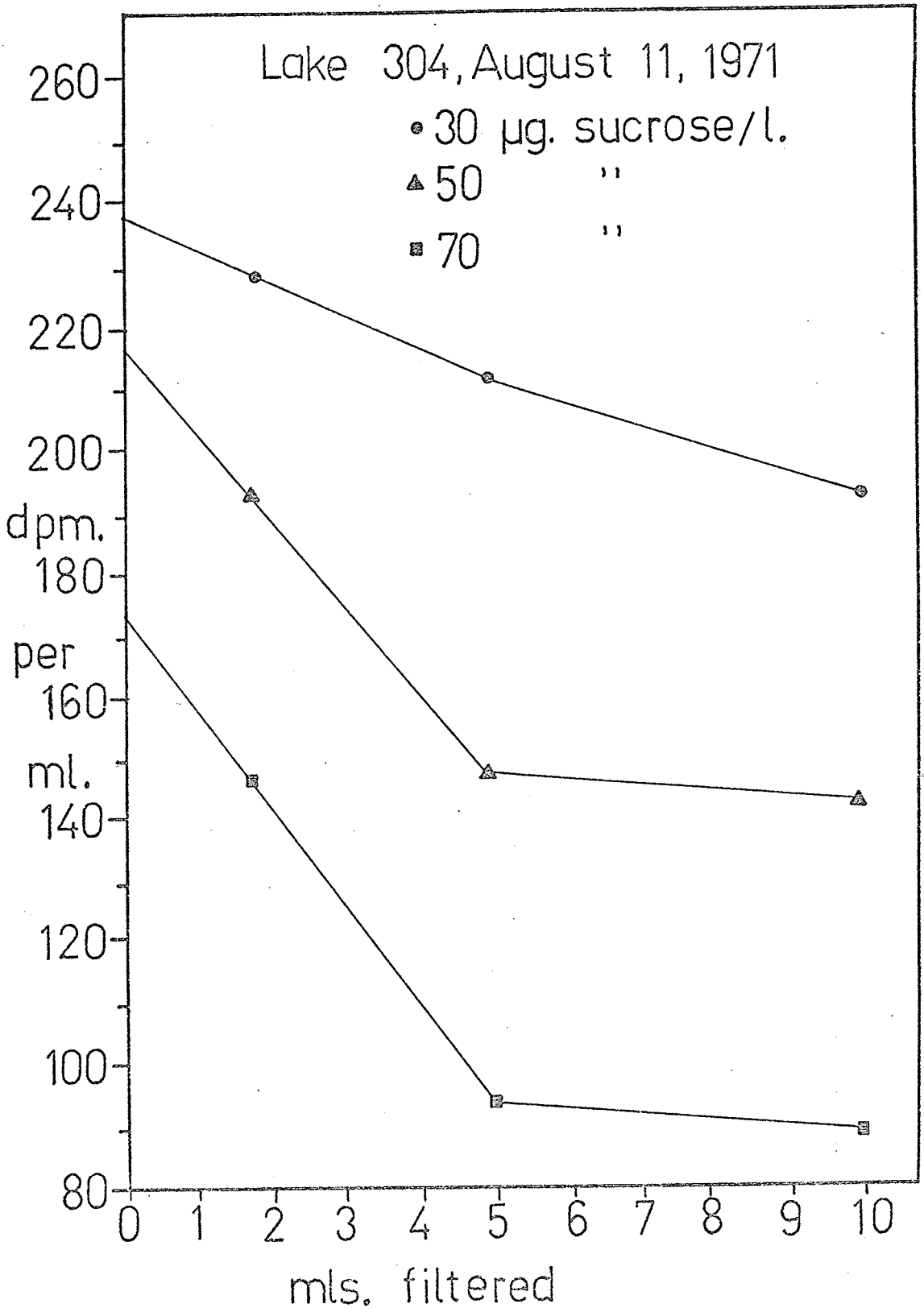
It is considered necessary to correct primary production and heterotrophic uptake data for filtration error if more accurate results are required; the ^{14}C-labelled material involved in this error is obviously a product of biological processes. In this study a correction factor for dpm in 5 ml of incubated sample was derived from $\frac{\text{dpm at y axis}}{\text{dpm at 5 ml}}$

TABLE 11

Filtration error: dpm per ml using hydrophobic edge filters.

ml filtered	dpm/ml	
	Hydrophobic edge	Control
50	420	468
25	479	488
10	538	561
5	569	593
2	639	581
1	678	607

Fig. 13. Filtration error, epilimnion of Lake 304, August 11, 1971.

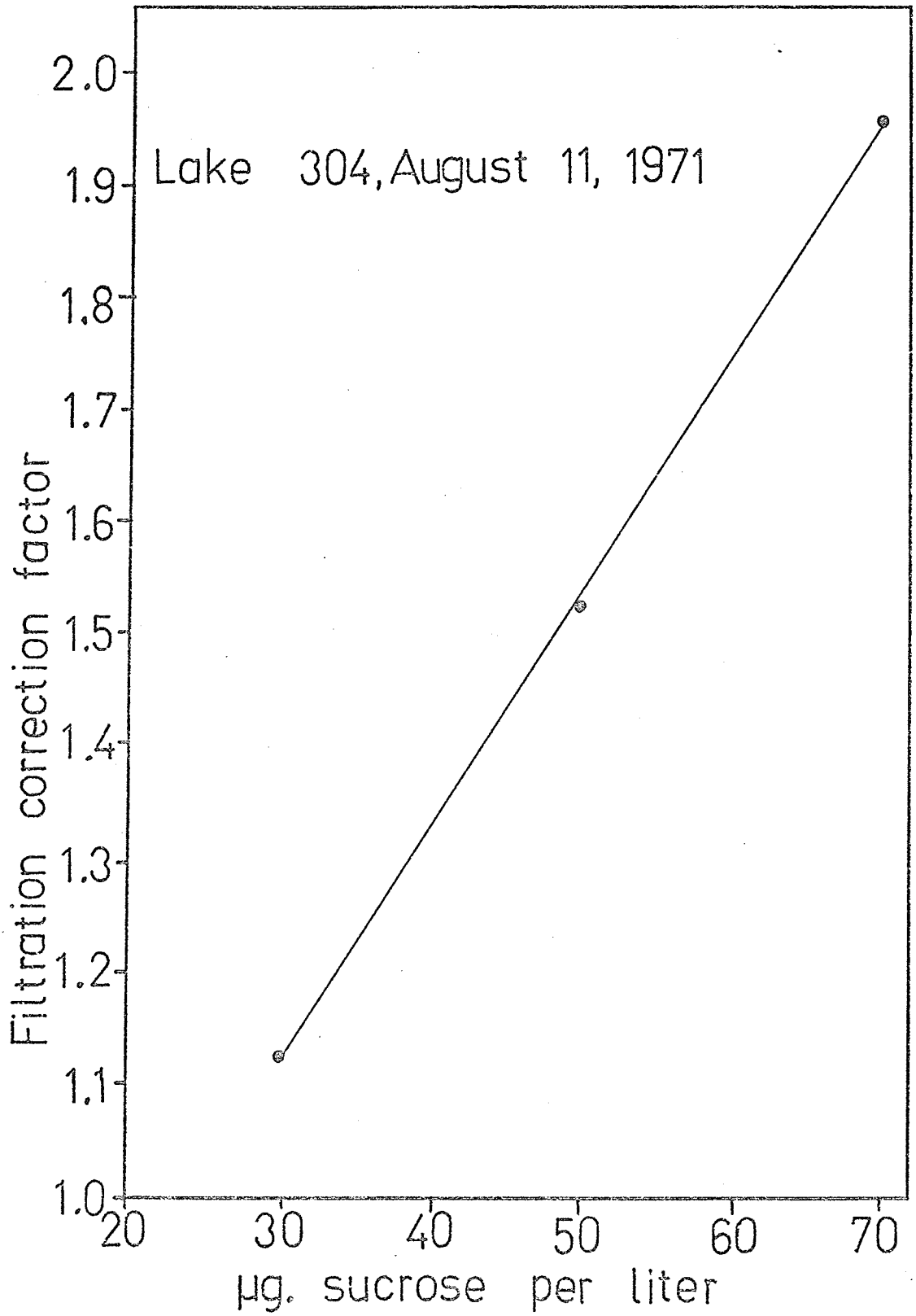

Lake 304, August 11, 1971
• 30 µg. sucrose/l.
▲ 50 "
■ 70 "
260
240
220
200
dpm.
180
per
160
ml.
140
120
100
80
0 1 2 3 4 5 6 7 8 9 10
mls. filtered

by plotting dpm/ml against ml filtered and extrapolating linearly to the y-axis (Fig. 13); this, however, is only an approximation.

However, these experiments do not clearly distinguish between two possible causes of filtration error, namely, cell breakage on vacuum filtration with loss of ^{14}C-labelled contents as proposed by Arthur and Rigler (58), and retention of large organic ^{14}C-molecules (probably by-products of microbial metabolism and photosynthesis) by membrane filters as suggested by Nalewajko and Lean (61). Some further observations may serve to clarify this problem.

During primary productivity studies on Shield lakes in the Experimental Lakes Area, filtration error was greatest during spring and fall (51). This could be due either to rupturing during filtration of the fragile phytoplankton species which are dominant in spring and fall or to increased excretion per cell of fixed carbon when phytoplankton numbers are low (62). On one occasion analysis of heterotrophic utilization of sucrose in Lake 304 yielded no filtration error. This corresponded to a phytoplankton population consisting mainly of Scenedesmus and Cryptomonas species which have strong cell walls and are not likely to burst on membrane filtration; only a few fragile Chrysochromulina cells were present (63). Though this appears to indicate a filtration error due to cell breakage, a clear conclusion cannot be drawn without analysis of extracellular products of metabolism. The filtration error associated with heterotrophic uptake was found to increase as substrate metabolized increased (Fig. 14); this observation supports both theories.

Fig. 14. Filtration correction factor, epilimnion of Lake 304, August 11, 1971.


Lake 304, August 11, 1971
Filtration correction factor
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
20
30
40
50
60
70
µg. sucrose per liter

The most conclusive evidence supporting the work of Nalewajko and Lean (61) was obtained during measurement of adenosine triphosphate (ATP) in several lakes in the Experimental Lakes Area (64). ATP in natural waters is present only in living cells. Concentration of ATP was obtained through the same filtration techniques employed in this study; it did not exhibit an increase in concentration per ml with decreasing volume filtered, that is to say, no filtration error was found. It can be concluded that cell breakage and loss of contents on vacuum filtration is not significant and that the explanation for filtration error advanced by Nalewajko and Lean is the most acceptable current theory.

APPENDIX III

Scintillation fluors

Bray's Fluor (46)

Naphthalene		60 g
PPO*		4 g
POPOP+		0.2 g
Methanol (absolute)		100 ml
Ethylene glycol		20 ml
p-Dioxane	to make	1 liter

* 2,5 diphenyloxazole

+1,4-di-2-(5-phenyloxazolyl) benzene

PPO was obtained from Amersham/Searle, POPOP from Nuclear Chicago, and naphthalene (scintillation grade) from Packard. Ethylene glycol, methanol and p-dioxane were obtained from Fisher Scientific.

Schindler's Fluor (47)

Naphthalene		100 g
PPO		7 g
Dimethyl-POPOP*		0.3 g
p-Dioxane	to make	1 liter

*2,2,p-phenylenebis(5 phenyloxazole) obtained from Nuclear Chicago.

Woeller's Fluor (56)

A	Phenethylamine		27 ml
	Methanol (absolute)		27 ml
	PPO		500 mg
	POPOP		10 mg
	Toluene	to make	100 ml

Woeller's Fluor (56) Cont'd

B	PPO		5 g
	POPOP		100 mg
	Toluene	to make	1 liter

Phenethylamine (scintillation grade) was obtained from Packard, and toluene was obtained from Fisher Scientific.

REFERENCES

1. Johnson, W.E. and Vallentyne, J.R. 1971. Rationale, background, and development of experimental lakes studies in northwestern Ontario. J. Fish. Res. Bd. Canada. 28: 123-128.

2. Brunskill, G.J. and Schindler, D.W. 1971. Geography and bathymetry of selected lake basins, Experimental Lakes Area, northwestern Ontario. J. Fish. Res. Bd. Canada. 28: 139-155.

3. Lowdon, J.A. and Blake, W., Jr. 1968. Geological survey of Canada: radiocarbon dates VII. Can. Geol. Surv. Pap. 68-2 Part B. Also in Radiocarbon 10: 207-245 (1968).

4. Vollenweider, R.A. 1968. Scientific fundamentals of the eutrophication of lakes and flowing waters with particular reference to nitrogen and phosphorus as factors in eutrophication. Tech. Rept. to the Org. Econ. Coop. and Development, Paris. DAS/CSI/68, 27: 1-182.

5. Parsons, T.R. and Strickland, J.D.H. 1962. On the production of particulate organic carbon by heterotrophic processes in seawater. Deep-Sea Res. 8: 211-222.

6. Wright, R.T. and Hobbie, J.E. 1965. The uptake of organic solutes in lake water. Limnol. Oceanogr. 10: 22-28.

7. Williams, P.J. LeB and Askew, C. 1968. A method of measuring the mineralization by micro-organisms of organic compounds in sea-water. Deep-Sea Res. 15: 365-375.

8. Hobbie, J.E. and Crawford, C.C. 1969. Respiration corrections for bacterial uptake of dissolved organic compounds in natural waters. Limnol. Oceanogr. 14: 528-532.

9. Harrison, M.J., Wright, R.T. and Morita, R.Y. 1971. Method for measuring mineralization in lake sediments. Appl. Microbiol. 21: 698-702.

10. Henrici, A.T. 1936. Studies on freshwater bacteria. III. Quantitative aspects of the direct microscopic method. J. Bacteriol. 32: 265.

11. Jannasch, H.W. and Jones, G.E. 1959. Bacterial populations in sea water as determined by different methods of enumeration. Limnol. Oceanogr. 4: 128-139.

12. Kriss, A.E. 1963. Marine Microbiology (Deep Sea). (Oliver and Boyd, Edinburgh and London, 536 pp.).

13. Steeman-Nielsen, E. 1952. The use of radio-active carbon (C^{14}) for measuring organic production in the sea. J. Cons. int. Explor. Mer 18: 117-140.

14. Fruton, J.S. and Simmonds, S. 1958. General Biochemistry. (Wiley and Sons, New York, 1077 pp.)

15. Dowd, J.E. and Riggs, D.S. 1965. A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations. J. Biol. Chem. 240: 863-869.

16. Caperon, J. 1967. Population growth in micro-organisms limited by food supply. Ecology 48: 715-721.

17. Hamilton, R.D. and Preslan, J.E. 1970. Observations on the continuous culture of a planktonic phagotrophic protozoan. J. Exp. Mar. Biol. Ecol. 5: 94-104.

18. Vaccaro, R.F. and Jannasch, H.W. 1966. Studies on heterotrophic activity in seawater based on glucose assimilation. Limnol. Oceanogr. 11: 596-607.

19. Vaccaro, R.F. 1969. The response of natural microbial populations in seawater to organic enrichment. Limnol. Oceanogr. 14: 726-735.

20. Vaccaro, R.F. and Jannasch, H.W. 1967. Variation in uptake kinetics for glucose by natural populations in seawater. Limnol. Oceanogr. 12: 540-542.

21. Hamilton, R.D. and Preslan, J.E. 1970. Observations on heterotrophic activity in the eastern tropical Pacific. Limnol. Oceanogr. 15: 395-401.

22. Williams, P.J. LeB. 1971. On the question of the validity of using simple kinetic equations to study natural aquatic populations. Research on the Marine Food Chain Progress Report, July 1970-June 1971, Institute of Marine Resources, University of California.

23. Hamilton, R.D. and Austin, K.E. 1967. Assay of relative heterotrophic potential in the sea: the use of specifically labelled glucose. Can. J. Microbiol. 13: 1165-1173.

24. Hobbie, J.E., Crawford, C.C. and Webb, K.L. 1968. Amino acid flux in an estuary. Science, 159: 1463-1464.

25. Hobbie, J.E. 1970. Heterotrophic bacteria in aquatic ecosystems; some results of studies with organic radioisotopes. pp. 181-194 in J. Cairns (ed.) The structure and function of freshwater microbial communities, Virginia Polytechnic Institute.

26. Hobbie, J.E. and Wright, R.T. 1965. Bioassay with bacterial uptake kinetics: glucose in freshwater. Limnol. Oceanogr. 10: 471-474.

27. Wright, R.T. and Hobbie, J.E. 1966. Use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology 47: 447-464.

28. Allen, H.L. 1968. Acetate in freshwater: natural substrate concentrations determined by dilution bioassay. Ecology 49: 346-349.

29. Siegel, A. and Degens, E.T. 1966. Concentration of dissolved amino acids from saline waters by ligand-exchange chromatography. Science 151: 1098-1101.

30. Williams, P.J. LeB. 1971. A study of heterotrophic activity in a near-shore station. Research on the Marine Food Chain Progress Report, July 1970-June 1971, Institute of Marine Resources, University of California.

31. Vallentyne, J.R. and Whittaker, J.R. 1956. On the presence of free sugars in filtered lake water. Science 124: 1026-1027.

32. Whittaker, J.R. and Vallentyne, J.R. 1957. On the occurrence of free sugars in lake sediment extracts. Limnol. Oceanogr. 2: 98-110.

33. Andrews, P. and Williams, P.J. LeB. 1971. Heterotrophic utilization of dissolved organic compounds in the sea. III. Measurement of the oxidation rates and concentration of glucose and amino acids in sea water. J. Mar. Biol. Assn. U.K. 51: 111-125.

34. Hicks, S.E. and Carey, F.C. 1968. Glucose determination in natural waters. Limnol. Oceanogr. 13: 361-363.

35. Vaccaro, R.F., Hicks, S.E., Jannasch, H.W. and Carey, F.G. 1968. The occurrence and role of glucose in seawater. Limnol. Oceanogr. 13: 356-360.

36. Samejima, H. and Meyers, J. 1958. On the heterotrophic growth of Chlorella pyrenoidosa. J. Gen. Microbiol. 18: 107-117.

37. Sloan, P.R. and Strickland, J.D.H. 1966. Heterotrophy of four marine phytoplankton at low substrate concentrations. J. Phycol. 2: 29-32.

38. Hellebust, J.A. and Guillard, R.R.L. 1967. Uptake specificity for organic substrates by the marine diaton Melosira nummuloides. J. Phycol. 3: 136-139.

39. Tolbert, N.E. and Zill, L.P. 1956. Excretion of glycollic acid by algae during photosynthesis. J. Biol. Chem. 222: 895-906.

40. Hellebust, J.A. 1965. Excretion of some organic compounds by marine phytoplankton. Limnol. Oceanogr. 10:192-206.

41. Wright, R.T. 1970. Glycollic acid uptake by planktonic bacteria. pp. 521-536 in D.W. Hood (Ed.) Symposium on organic matter in natural waters, held at the University of Alaska, Sept. 2-4, 1968. Institute of Marine Science, 1970.

42. Storch, T.A. 1970. Production of extracellular dissolved organic carbon by phytoplankton and its utilization by bacteria. Ph.D. thesis, University of Michigan.

43. Sorokin, Y.I. 1970. Determination of the activity of heterotrophic microflora in the ocean using C^{14}-containing organic matter. Microbiology 39: 133-138.

44. Satomi, Y. 1966. Physiological significance of carbon sources in fertilized fish ponds. Bull. of Freshwater Fisheries Res. Lab. 15: 99-104.

45. Warren, C.E., Wales, J.H., Davis, G.E. and Doudoroff, P. 1964. Trout production in an experimental stream enriched with sucrose, J. Wildlife Management 28: 617-666.

46. Bray, G.A. 1960. A simple efficient liquid scintillator for counting aquaeous solutions in a liquid scintillation counter. Anal. Biochem. 1: 279-285.

47. Schindler, D.W. 1966. A liquid scintillation method for measuring carbon-14 uptake in photosynthesis. Nature 211: 844-845.

48. Wood, L.W. 1971. Heterotrophic uptake of disaccharides by aquatic microbial communities. American Society of Limnology and Oceanography Conference, Winnipeg, 1971.

49. Roseman, S. 1968. The transport of carbohydrates by a bacterial phosphotransferase system. J. Gen. Physiol. 54 (Pt. 2): 138s-184s.

50. Wood, L.W. Unpublished data.

51. Schindler, D.W. Personal communication.

52. Schindler, D.W., Lean D.R.S. and Fee, E.J. 1972. Nutrient cycling in freshwater ecosystems. Proceedings, IBP Symposium on World Ecosystems, Seattle, Washington, Sept. 1972 (in press).

53. Thompson, B.M. and Hamilton, R.D. 1972. Problems in heterotrophic uptake methodology. Proceedings, U.S.-Japan conference on Marine Microbiology, University of Maryland, August 1972 (in press).

54. Schindler, D.W., Schmidt, R.V. and Reid, R.A. 1972. Acidification and bubbling as an alternative to membrane filtration in determining phytoplankton production by the ^{14}C method. J. Fish. Res. Bd. Canada. 29: 1627-1631.

55. Merowitz, D. Personal communication.

56. Woeller, F.H. 1961. Liquid scintillation counting of $^{14}CO_2$ with phenethylamine. Anal. Biochem. 2: 508-511.

57. Georgi, P. and Lober, M. 1965. Beobachtungen über das instabile Verhalten von ^{14}C-Phenyläthylamincarbamat in Flüssigkeitss-zintillatorgemischen. Acta Biologica et Medica Germanica 14: 91-93.

58. Arthur, C.R. and Rigler, F.H. 1967. A possible source of error in the ^{14}C method of measuring primary productivity. Limnol. Oceanogr. 12: 121-126.

59. Schindler, D.W. and Holmgren, S.K. 1971. Primary production and phytoplankton in the Experimental Lakes Area, northwestern Ontario, and other low-carbonate waters, and a liquid scintillation method for determining ^{14}C activity in photosynthesis. J. Fish. Res. Bd. Canada 28: 189-201.

60. Berman, T. and Williams, P.J. LeB. 1971. Notes on the methodology of the radiocarbon technique for studying algal productivity. Research on the Marine Food Chain Progress Report, July 1970-June 1971, Institute of Marine Resources, University of California.

61. Nalewajko, C. and Lean, D.R.S. 1972. Retention of dissolved compounds by membrane filters as an error in the ^{14}C method of primary production measurement. J. Phycol. 8: 37-43.

62. Nalewajko, C., Chowdhuri, N. and Fogg, G.E. 1963. Excretion of glycollic acid and the growth of a planktonic Chlorella, pp 171-183 in Studies on microalgae and photosynthetic bacteria, Japanese Society of Plant Physiology, Tokyo.

63. Kling, H. Personal communication.

64. Rudd, J.W.M. and Hamilton, R.D. The measurement of adenosine triphosphate (ATP) in two pre-Cambrian Shield lakes of northwestern Ontario. J. Fish. Res. Bd. Canada (in press).