DIURNAL CHANGES IN THE HETEROTROPHIC UPTAKE OF GLYCOLATE AND GLUCOSE IN TWO LAKES

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A B S T R A C T

A study was made of diurnal changes in the heterotrophic uptake kinetics of both ^{14}C-glucose and ^{14}C-glycolate in two small stratified lakes, Lake Rotomanuka in New Zealand and Lake Fryxell in Antarctica. The purpose of the investigation was to study the effect that diurnal changes in the extracellular release of certain substrates by algae might have on the uptake of those substrates by aquatic microheterotrophs. The heterotrophic uptake technique of Parsons and Strickland (1961) was modified to provide a technique which was more convenient for field-work. In an effort to exclude the effects of migration and heterogeneous population distribution on substrate uptake, the microbial population in each lake was observed using a combination of (a) direct counts of bacteria (using acridine orange staining and epifluorescent microscopy); (b) measurement of chlorophyll *a* and bacteriochlorophyll and (c) measurement of the reduction of INT dye. The natural concentration of glycolate was measured on some occasions using a modification of the technique of Shah and Wright (1974).

In Lake Rotomanuka changes in the uptake kinetics of glycolate were observed within three out of four 24 hour periods. On two occasions these observed changes resulted in significant differences ($P<0.05$) between measurements at different times of day of each of the three kinetic parameters, V_{max} (the theoretical maximum uptake rate), T_t (substrate turnover time) and $(K_T + S_n)$ (the sum of the uptake rate constant and the naturally occurring substrate concentration). On the other occasion the apparent changes in uptake kinetics were not amenable to statistical analysis using the Michaelis-Menten kinetic model. The uptake kinetics for glucose were observed to change over one out of two 24 hour periods but the changes were not amenable to statistical analysis. For neither glucose nor glycolate was it possible to conclude that the observed kinetic changes were diurnally cyclic and/or stimulated by diurnal changes in the concentrations of these substrates.

At a depth of 9m in Lake Fryxell, uptake kinetics were not amenable to analysis by Michaelis-Menten kinetics. However, obvious changes occurred in the partitioning between biosynthesis and respiration to $^{14}CO_2$ of ^{14}C-activity, from both glucose and glycolate. These changes were not correlated with time of day. Some evidence is provided by the other techniques used that migration of microbial populations about the sampled depth was occurring. Aside from the aims of this thesis, a

bacteriochlorophyll peak was found at the oxycline of Lake Fryxell and a study of the microstratification of organisms about the oxycline revealed that changes in the positions of population peaks occurred both within a time scale of 24 hours (for algae) and on a time scale of days (for photosynthetic bacteria) at the time of study (December-January).

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C O N T E N T S

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LIST OF ABBREVIATIONS

(Only abbreviations which are not in common usage or which might lead to confusion are included.)

A	The concentration of labelled and unlabelled substrate added to a water sample.
Bq	Becquerel - unit of radioactivity (1 Bq = 1 disintegration per second).
Ci	Curie - unit of radioactivity (1 μCi = 2.2×10^6 disintegrations per minute).
DHN	2,7-dihydroxynaphthalene, colourimetric reagent for glycolate analysis.
ETS	Electron transport system.
INT	2-(*p*-iodophenyl)3(*p*-nitrophenyl)5 phenyl tetrazolium chloride.
K_T	The rate constant for uptake.
PDOC	Photosynthetically derived, dissolved organic carbon.
P.E.R.	Percent extracellular release (extracellular release as a fraction of carbon fixation).
S_n	Naturally occurring concentration of a substrate.
t/f	Incubation time divided by the fraction of added ^{14}C-substrate which is taken up.
T_t	Turnover time of a substrate. The time required for complete uptake of naturally occurring substrate at current rate of uptake, assuming no enrichment with added substrate.
V_{max}	The theoretical maximum possible uptake rate of an uptake system.
μgCl^{-1}	Micrograms of carbon per litre.
μgl^{-1}	Micrograms per litre.

Chapter 1

INTRODUCTION

AND

LITERATURE REVIEW

1.1 INTRODUCTION

The importance of the role of bacteria in the recycling of growth-limiting nutrients, principally carbon, phosphorus and nitrogen, in aquatic ecosystems, has long been recognised. The fulfillment of this role, analogous to the same situation in soil, is often referred to, perhaps inadequately, as the "decomposer" stage of mineral cycling. Inadequate because, as with the discovery of the rhizosphere in soil, it has been discovered in aquatic ecosystems that not all bacterial heterotrophy is saprophytic. A proportion of nutrient cycling, particularly of carbon, is achieved by bacterial utilisation of compounds excreted by healthy, living primary producers; plant roots in soil, planktonic algae in pelagic waters. In the rhizosphere this flow of nutrients is of benefit to both parties involved. Whether this is so in aquatic ecosystems, has yet to be determined. Also to be determined is exactly what proportion of carbon flow involves excretion by photosynthesising phytoplankton cells as opposed to lysis and decomposition of dead cells.

An area of microbial ecology receiving a lot of attention at the moment is based on the realisation that conditions in laboratory cultures of organisms, whether they be chemostat or batch cultures, do not adequately model conditions outside the laboratory. One important aspect of this is the supply of substrate with respect to time. This supply may be regularly or randomly fluctuating in nature as opposed to constant, or decreasing in a regular fashion in the laboratory. The adaption of bacteria to a regime of fluctuating substrate supply is now coming under close scrutiny (Koch, 1971; Tempest and Neijssel, 1978; Dawes, 1976; Shilo (Ed), 1978).

The connection between these two areas of research was proposed by Saunders (1969) and Saunders and Storch (1971). First, it was proposed that the diurnal pattern of phytoplankton photosynthesis, which peaks during the day and is zero at night, is closely coupled to a similar pattern in phytoplankton excretion and that consequently those heterotrophic bacteria which utilise the excretion products are subject to an alternating on/off supply on a 24 hour basis, superimposed on the already recognised medium-to-long-term changes caused by weather and season. Second, it was proposed that the alternating supply of carbon substrate would initiate changes in the

capacity of heterotrophic populations to take up those substrates involved, either at a population level by population growth or decline, or at an individual level by induction or repression of the enzyme systems involved in substrate uptake and metabolism. Evidence to support the first proposal was obtained from an artificial model system in which glucose was added to an enclosed body of lake water over a 24 hour period in a pattern representative of previously measured phytoplankton excretion. Uptake of ^{14}C-labelled glucose at a single tracer level concentration followed a similar pattern to the addition pattern but lagged behind it by about three hours, to peak in the late afternoon. "Tracer level" refers to a low enough concentration of added substrate to be insignificant compared with *in situ* concentration.

Further evidence for this proposal was provided by Hanson and Snyder (1980) who measured *in situ* glucose concentration on a diurnal basis in a salt marsh estuary and found that on a significant number of occasions glucose concentration was greatest in the afternoon and decreased in the evening to a minimum at dawn. McKinley and Wetzel (1979) measured the uptake of tracer levels of ^{14}C-glucose in lake water at dawn, mid-day and late afternoon and found a general pattern of increasing uptake throughout the day. Gocke (1970) reported that the concentration of free amino acids decreased during the hours of darkness in two lakes. Williams and Yentsch (1976) found that in oligotrophic coastal seawater, the uptake of a mixture of ^{14}C-labelled amino acids at tracer levels was lower in the hours of darkness (but with a marginal significance of 5-10% probability).

Although these results indicate that some substrates can change in concentration on a regular diurnal basis, they do not support the second proposal of Saunders that heterotrophic bacteria adjust their ability to use these substrates. Uptake of a ^{14}C-labelled substrate at tracer levels is, in theory, as much dependent on the magnitude of *in situ* concentration as it is on the metabolic capacity of the heterotrophic population. In practice, Meyer-Reil *et al* (1979) found that the uptake of tracer levels of ^{14}C-glucose and ^{14}C-fructose varied over a 24 hour period at two sites in the Baltic Sea but that these changes were paralleled by changes in the *in situ* concentrations of glucose and fructose.

The purpose of this thesis was to examine the extent to which heterotrophic populations in lakes adjust their capacity to take up substrates associated with algal excretion, on a diurnal basis. The technique used was the heterotrophic uptake technique (see section 1.2), in which uptake of a ^{14}C-labelled substrate is measured over a range of concentrations yielding so-called "kinetic" information on the type of uptake system involved and the extent to which it is "switched on".

It was hoped that research along these lines would help to answer the following questions:

(a) Is algal excretion in lakes of sufficient magnitude compared with other sources of carbon substrate, to initiate changes in the uptake kinetics of heterotrophic microbial populations in response to changing excretion rates?

(b) Are changes in algal excretion rates of sufficient magnitude, on a diurnal basis, to initiate changes in the uptake kinetics of heterotrophic microbial populations?

(c) What strategies, in terms of changing uptake kinetics, are adopted by members of the heterotrophic, microbial population to deal with diurnally fluctuating substrate levels?

1.2 HETEROTROPHIC UPTAKE

1.2.1 Introduction

In 1961 Parsons and Strickland found that when ^{14}C-labelled substrates such as glucose or acetate, were added to seawater, over a range of concentrations, the rate of incorporation of ^{14}C-label into microbial biomass, when plotted against the concentration of added substrate, yielded a rectangular hyperbola. Parsons and Strickland assumed that the saturation kinetics they were measuring were those of the enzyme for the initial, rate-limiting step in the metabolism of the substrate concerned and analysed these kinetics using the Michaelis-Menten equation to produce a characteristic rate constant for uptake.

1.2.2 Michaelis-Menten Kinetics

The Michaelis-Menten equation relates the substrate concentration, the reaction velocity and the theoretical maximum attainable reaction velocity at an infinite substrate concentration, for a single substrate enzyme-mediated reaction (Lehninger, 1975). It is written as

$$V_0 = \frac{V_{max}\ (S)}{K_m + (S)} \qquad \text{equation 1}$$

Where V_0 is initial reaction velocity

V_{max} is maximum attainable reaction velocity

(S) is concentration of reaction substrate

K_m is a constant dependent on the particular enzyme and on reaction conditions.

Equations of this form produce rectangular hyperbolas such as those seen in Figure 1A. Enzyme-mediated reactions involving single substrates follow the following reaction sequence:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_1}{\rightleftharpoons}} E + P \qquad \text{equation 2}$$

Where E is the enzyme

S is the substrate

P is the product

and k_1, k_{-1}, k_2 and k_{-2} are rate constants for each respective step.

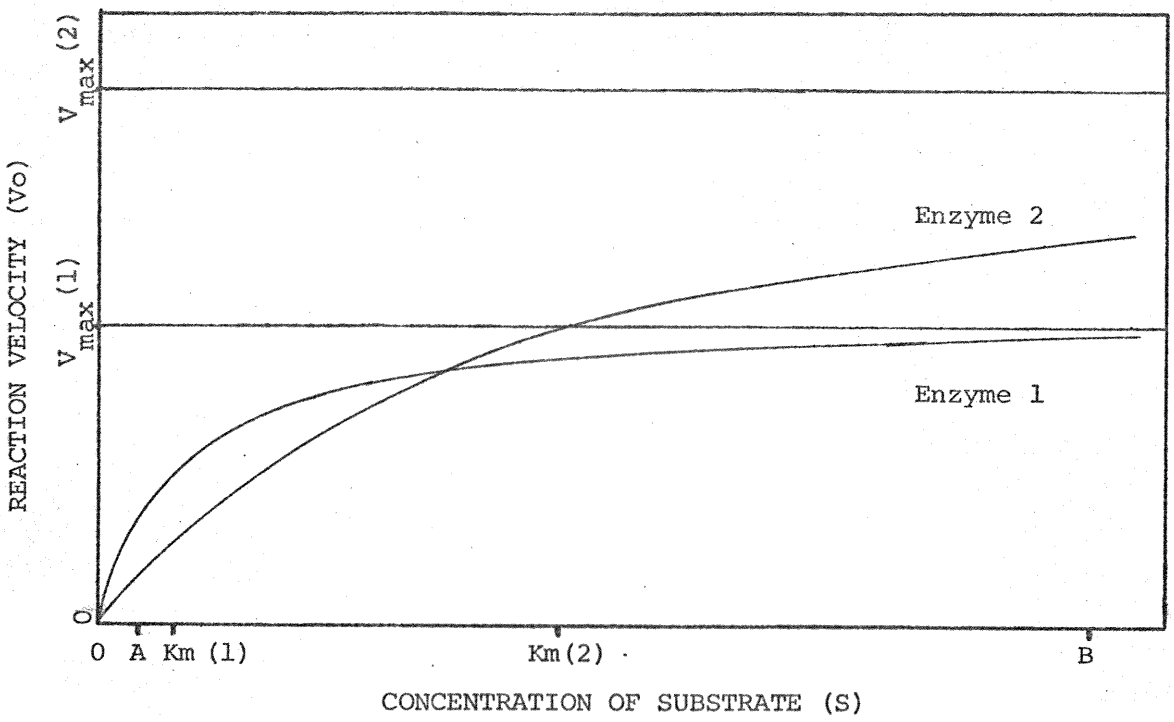


Figure 1/1A - Graph of Reaction Velocity (Vo) vs. Substrate Concentration (S) for two Enzyme Mediated Reactions involving Single Substrates

Arbitrary units on each axis

See section 1.2.2 for discussion

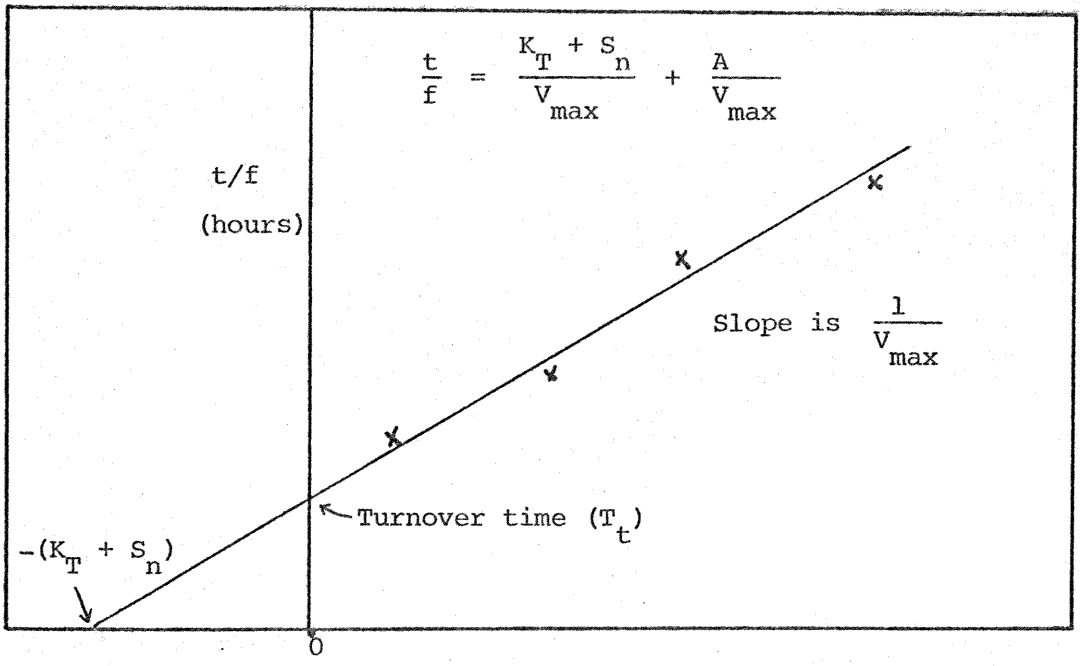


Figure 1/1B - Graph of the Relationship between t/f (incubation time/fraction of added substrate which is taken up) and A (the concentration of added substrate)

See section 1.2.3 for discussion

In the derivation of the Michaelis-Menten equation k_{-2} is ignored and it is assumed that V_0, the initial reaction velocity is measured before any significant amount of product is formed.

Returning to figure 1A it can be seen that, initially reaction velocity increases almost linearly with substrate concentration but that the rate of increase gets less with increasing concentration. This is because of the finite time required for the formation and dissociation of the enzyme-substrate complex. Initially reaction velocity is limited by the time taken for the enzyme molecule to encounter a substrate molecule. At high substrate concentrations reaction velocity is limited by the formation/dissociation time of the enzyme-substrate complex. As this time is a characteristic of the enzyme and its environment (with respect to temperature, pH, cation availability etc.), under constant conditions V_{max} is proportional to enzyme concentration. If the enzyme is part of a bacterium then V_{max} is dependent on the number of enzyme molecules per cell and the concentration of cells.

Another important parameter of the Michaelis-Menten equation is Km. In the special case when

$$V_0 = \tfrac{1}{2} V_{max} \qquad \text{equation 3}$$

rearrangement of the equation yields the result:

$$K_m = (S) \qquad \text{equation 4}$$

Thus, K_m is equal to the substrate concentration at which the reaction velocity is half of its maximum attainable velocity. As such, it can be used as an indicator of the ability of the enzyme to catalyse the reaction at low substrate concentrations.

Of two enzymes with the same V_{max}, the enzyme with the lower K_m will have a higher affinity for the substrate and its use in a reaction system will result in higher values of V_0 at low substrate concentrations.

An examination of the two sets of uptake kinetics in figure 1A reveals that at the substrate concentration labelled A enzyme (1) yields a higher reaction rate than enzyme (2). At higher concentrations however, for example at the point labelled B, enzyme (2) is responsible for a faster reaction rate. In terms of the uptake of carbon substrates in aquatic ecosystems it follows that if two organisms have different

uptake kinetics, one with kinetics corresponding to those of enzyme (1) and the other with kinetics like those of enzyme (2), then in a carbon limited environment, the first organism will have a competitive advantage at low substrate concentration and the other will have the advantage at high substrate concentration.

1.2.3 The Application of Michaelis-Menten Kinetics to the Uptake of Carbon Substrates : Theoretical Considerations

In order to measure the uptake kinetics of a given substrate by aquatic microbial populations, known concentrations of labelled substrate are added to the water. However, unless the natural concentration of substrate is known, and this is often very difficult or laborious to measure, the final concentration, or range of concentrations is unknown. Using the terminology of Hobbie and Rublee (1977), the Michaelis-Menten equation can be rewritten as

$$V_{(S_n + A)} = \frac{S_n + A}{K_T + S_n + A} \qquad \text{equation 5}$$

where $V_{(S_n + A)}$ is the uptake rate of added and natural substrate

V_{max} is the maximum value of uptake rate

K_T is a constant equivalent to the Michaelis-Menten constant. The T subscript is used to denote transport into a cell.

S_n is the natural substrate concentration and

A is the added substrate concentration.

Wright and Hobbie (1965, 1966) employed a linear transformation of this equation to enable calculation of the kinetic parameters. First, uptake rate was expressed in terms of the incubation time (t) and the fraction of labelled substrate which was taken up (f).

$$V_{(S_n + A)} = (f/t)\ (S_n + A) \qquad \text{equation 6}$$

which is equivalent to

$$t/f = \frac{S_n + A}{V_{(S_n + A)}} \qquad \text{equation 7}$$

Second, each side of equation 5 was divided by $(S_n + A)$, then inverted:

$$\frac{(S_n + A)}{V_{(S_n + A)}} = \frac{(S_n + A) + K_T}{V_{max}} \qquad \text{equation 8}$$

Finally, equations 7 and 8 were combined to give

$$t/f = \frac{K_T + S_n}{V_{max}} + \frac{A}{V_{max}} \qquad \text{equation 9}$$

which is the formula of a linear relationship between A and t/f, with a slope of $1/V_{max}$ and an x-intercept at $-(K_T + S_n)$. (See figure 1B). The y-intercept is t/f at zero added substrate. This is the time it would take to utilise all of the naturally occurring substrate at its initial rate of uptake or in other words it is the turnover time of the substrate (T_t).

Without knowing the value of S_n it is not possible to calculate the actual uptake rate in the water body. Nor is it possible to determine the relative contributions of K_T and S_n to a value of $(K_T + S_n)$. Therefore it is necessary to remember, when comparing different sets of kinetics, that differences in this term may be due to differences in either K_T or in S_n.

The linear transformation employed by Wright and Hobbie was based on a modification of the Lineweaver-Burke equation (Lehninger, 1973). In future, I will refer to the resulting graph of t/f against A as a modified Lineweaver-Burke plot.

1.2.4 Problems Associated with the Use of Michaelis-Menten Theory to Interpret the Uptake Kinetics of Natural Populations

The Michaelis-Menten equation describes the kinetics of a single enzyme-single substrate reaction under a specified set of conditions. These conditions are:

(a) the concentration of the reaction product or products is zero at all times;

(b) the reaction pathway described involves only one enzyme;

(c) the enzyme concentration remains constant;

(d) only one reaction pathway is involved.

Assuming the fulfillment of these conditions, a given reaction will only be described by a unique Michaelis-Menten equation if:

(e) other substrates for the reaction remain constant;

(f) the reaction environment, with respect to pH, ion concentration, temperature etc., remains constant.

Each of these conditions is discussed below in terms of its effect on the use of Michaelis-Menten kinetics to describe substrate uptake reactions in natural waters.

(a) The first condition for validity of the Michaelis-Menten equation is that product concentration is zero. Product concentration is not zero except in the ideal case when metabolism of the substrate is very much faster than its rate of entry into the cell. This has not yet been established as a general truth although Kay and Grunland (1969) found that the intracellular amino acid pool of *Pseudomonas aeruginosa*, when fed on amino acids, was very low. Intracellular amino acids are the products of the uptake of extracellular amino acids.

The alternative situation, in which metabolism forms an integral part of uptake kinetics is discussed in (b).

(b) The Michaelis-Menten equation describes a single enzyme-single substrate reaction, regarded in isolation. In a bacterial cell the uptake step is followed by other reaction pathways which utilise the substrate. These reactions, depending upon their rate relative to the uptake reaction, may affect the measured kinetics of uptake. There is not enough evidence available to warrant a generalisation to the effect that the measured kinetics of uptake of a single substrate by a single bacterial species can be attributed to the kinetics of a single enzyme. Evidence that this is sometimes the case is provided by Herbert and Kornberg (1976) who found that two strains of *E.coli*, grown aerobically in continuous culture under conditions of glucose limitation, utilised glucose at rates identical to those at which cells harvested from the chemostats transported ^{14}C-glucose. The inference was that glucose uptake was the rate limiting step in glucose utilisation by the cells and thus the measured uptake kinetics were actually those of the uptake step.

Krambeck (1979) produced a mathematical model for uptake by a sequential enzyme pathway with an arbitrary number of components. One finding from this model was that as the uptake rate increased, concentrations of intermediate metabolites increased and caused significant back reaction in the

enzyme chain, the result being that the approach to V_{max} with increasing substrate concentration was slower than that occurring with pure Michaelis-Menten kinetics. However, the conclusion was that Michaelis-Menten kinetics can provide an approximate fit if all of the opposing effects in an enzyme chain are balanced and that experimental design is a greater source of error than theoretical limitations.

(c) Unlike cell free enzyme systems, natural populations have the ability to increase enzyme concentration as a response to added substrate, by enzyme induction or population growth. This is not normally a problem if incubation times are kept short and the linearity of uptake with time is tested (Hobbie and Rublee, 1977) (Wright, 1971).

(d) Parallel enzyme systems provide a departure from the single enzyme condition. Geesey and Morita (1979) found that the Lineweaver-Burke linearisation of the uptake kinetics of arginine for a psychrophilic marine bacterium was characterised by two distinct linear sections. This they interpreted as meaning that the bacterium had two simultaneously occurring uptake pathways with different K_T values.

Another way in which multiple uptake pathways can occur for the same substrate is through the co-existence of different, heterotrophic organisms in the same water body. Stanley and Staley (1975) used autoradiography to demonstrate a range of uptake kinetics for acetate by different morphological types of bacteria in the same highly eutrophic oxidation pond. In one example, an unidentified elongated rod had a similar V_{max} per unit biomass to the easily identified prosthecate species, *Ancalomicrobium adetum*. It also had a five times smaller $(K_T + S_n)$.

Williams (1973) created a mathematical model for testing the error in the calculation of turnover time when the uptake kinetics of a large number of theoretical bacterial species are pooled to give population uptake kinetics. His conclusion

was that unless wide variations in the K_T values of sub-populations are encountered, the errors are acceptable, e.g. if the kinetics of 1000 subpopulations of bacteria with an order of magnitude range in their individual values of K_T and V_{max}, are summed, the error in calculating T_t, using an extrapolation from the linear portion of the modified Lineweaver-Burke plot discussed in section 1.2.3, will be 44%, which would be reduced to about 25% if the natural substrate concentration is about 1/3 of the observed value of K_T. The error in V_{max} would be zero and in K_T negligible. Based on the work of Stanley and Staley and also on that of Hamilton, Morgan and Strickland (1966) who measured the glucose uptake kinetics of some marine bacteria, a ten-fold range in K_T values in a natural water sample seems reasonable.

Vaccarro and Jannasch (1967) found sigmoidal uptake kinetics in oligotrophic oceanic samples which, when pre-incubated with glucose, the substrate used to measure kinetics, yielded saturation type kinetics. This was interpreted as a change from a heterogeneous population to a monodominant population, an interpretation which has been accepted by some more recent workers, e.g. Wright (1973), and used as evidence for a wider range of K_T values in oligotrophic waters than in eutrophic waters where saturation-type uptake kinetics are immediately apparent. This contention is supported by Gocke (1977) who found similar deviations from Michaelis-Menten theory to those predicted by Williams (1973) in oligotrophic ocean water. However, Krambeck (1979) notes that the sigmoid curves of Vaccarro and Jannasch (1967) can not be explained by a heterogeneous population effect and are more likely due to certain metabolic pathways needing threshold concentrations before they operate. The results of Stanley and Staley (1975) suggest that heterogeneous populations are just as likely to occur under eutrophic conditions as under oligotrophic conditions. To some extent the problem of heterogeneous populations can be minimised by keeping added substrate as low as possible to decrease the effect of uptake by systems with very high K_T values, particularly by algae without

transmembrane permease systems which, rely on diffusion through the membrane for uptake (Wright and Hobbie, 1965, 1966), (Wright, 1973).

(e) Natural waters contain a variety of different substrates which may be used simultaneously by a single uptake pathway. This introduces the problem of competitive inhibition. For example, Wright (1968) found that the presence of lactate at 100 μgl^{-1} could increase the $(K_T + S_n)$ of glycolate by 100 μgl^{-1} without changing the V_{max}. Crawford, Hobbie and Webb (1974) concluded that, although competitive inhibition of amino acid uptake by related amino acids did occur, it was unlikely to be important in natural waters because of the high levels needed for inhibition. For example, in the estuary studied, total dissolved amino acids ranged in concentration from 10-30 μgl^{-1} over a year but 1 mgl^{-1} glutamic acid increased T_t and $(K_T + S_n)$ of aspartic acid by a factor of only three. As Hobbie and Rublee (1977) point out, competitive inhibition affects only the measurement of $(K_T + S_n)$. T_t is changed by competitive inhibition but the measured value is still representative of actual turnover of the substrate in the water being studied. V_{max} is not changed. As V_{max} is likely to be a more important indicator of the induction/repression of enzyme systems, $(K_T + S_n)$ already being dependent on S_n, competitive inhibition will not unduly limit the use of uptake kinetics in this work.

(f) Measurement of the kinetics of isolated enzymes in a laboratory always involves the specification of reaction conditions, such as pH and cation concentration, as these affect enzyme-substrate association and dissociation. This is impossible in natural waters because of the variety of parameters which would need measuring and because of the microheterogeneity of the natural physiochemical environment. In a study of kinetic changes with time, which is the purpose of this thesis, it must be assumed that changes in kinetics are not due merely to a changing physical or chemical environment. Gross changes in physical and chemical parameters are unlikely over a 24 hour period in a small, well mixed lake.

They are more likely to be associated with the meeting of different water masses at a sampling site, as occurred in the work of Gocke (1975) in which case changes in microbial populations are likely to have more serious effects than changes in the physiochemical environment.

For the interpretation of (a), (b), (c) and (d), the attitude of reviews on the subject by Krambeck (1979) and Wright (1973) appears to be that if deviations from Michaelis-Menten kinetics are apparent, i.e. if the modified Lineweaver-Burke technique described in section 1.2.3 yields non-linear plots, then the results must be discarded. Otherwise they are usable. However, it is only fair to point out that this is not a universal view. Jannasch (1974) and Vaccarro and Jannasch (1967) suggest that even with a linear relationship it is unwise to extrapolate from that relationship without knowing the causes of linearity.

Although the effects of the uptake environment, (e) and (f), are not able to be assessed it is important to bear in mind that they may exist.

1.3 ALGAL EXTRACELLULAR RELEASE

This is not intended to be a complete review on the area of algal extracellular release as this is an extensive area which has been well reviewed by various authors. Fogg, Nalewajko and Watt (1965) provide a general review of the extracellular products of phytoplankton photosynthesis. Fogg (1971) covers release by freshwater algae, Hellebust (1974) and Raymont (1980) cover release by marine phytoplankton and Nalewajko (1977) discusses release by freshwater algae and bacteria and the subsequent utilisation of extracellular compounds by bacteria.

Extracellular release is defined by Nalewajko (1977) as "the liberation of soluble organic compounds by living organisms". This is as opposed to compounds released by "moribund" organisms or lysing cells. It is sometimes referred to as PDOC (photosynthetically derived dissolved organic carbon) (Wiebe and Smith, 1977).

A wide variety of compounds are selectively released, with different algae releasing a different range of compounds. The selectivity of release is suggested by the fact that many compounds known to occur within algal cells are never released (Nalewajko, 1977).

The range of excreted products can be loosely divided into three groups:

(i) miscellaneous compounds; vitamins, growth modifying substances, enzymes, phenolic substances, sex factors and toxins;

(ii) high molecular weight metabolic end products such as polypeptides and polysaccharides, which are often associated with capsular material;

(iii) the group of low molecular weight compounds such as the sugars, organic acids and amino acids.

This last group is that most relevant to this thesis in that the compounds involved are readily utilised by heterotrophic bacteria. In this context it would be pertinent to discuss the following:

(a) the extent to which low molecular weight carbon substrates are excreted;

(b) the extracellular release of glycolic acid, as an example of a low molecular weight excretory product;

(c) whether extracellular release of low molecular weight compounds particularly glycolate changes, or is likely to change, on a diurnal basis, and

(d) glucose, an example of a low molecular weight compound which probably occurs in natural waters largely as a result of processes other than extracellular release.

(a) The usual method for quantifying extracellular release by pure cultures or natural populations, is as follows. A sample of the algae is incubated in the light with added $NaH^{14}CO_3$. At the end of incubation the sample is gently filtered to remove the algal cells without disrupting them. The filtrate is then acidified and bubbled to remove the remaining $^{14}CO_2$. The radioactivity remaining in solution is taken to be due to extracellular products and the sum of this value and the radioactivity remaining on the filter is total CO_2 fixation. Extracellular release is frequently expressed as a fraction of CO_2 fixation; Percent Extracellular Release or P.E.R.

P.E.R. values are available for numerous experiments under a variety of conditions. Examples of quoted values are 7 to 50% and occasionally up to 95% in natural populations and pure cultures (Fogg, Nalewajko and Watt, 1965), 3 to 25% in pure cultures under artificial conditions (Hellebust, 1965), 1 to 40% for natural populations in a wide variety of ocean areas (Raymont, 1980), an average of 7% in coastal tropical waters (Williams and Yentsch, 1976) and 25 to 45% in coastal waters of the Baltic Sea (Larsson and Hagström, 1979).

Sharp (1977) has suggested that many of the high results obtained are experimental artifacts due to, among other things, damage of cells by filtration, organic contamination in the $NaH^{14}CO_3$ used and metabolic imbalances created in algal cells by incubating them under different conditions to those which they have been conditioned to. He further suggested, on the basis of his own work, that P.E.R. values of from 1-5% are more realistic. Fogg (1977) successfully defended the early work by Fogg, Nalewajko and Watt against these criticisms. Further support for the theory that extracellular release in natural waters is greater than 1-5%, is provided by Mague,

Friberg, Hughes and Morris (1980) who studied the time course of extracellular release by natural marine phytoplankton populations under different lighting conditions in an effort to recognise experimental artifacts. They concluded that P.E.R. values of 6% to 50% are reasonable, but warn that P.E.R. values are misleading in that high P.E.R. values often reflect low production rather than high release. This is supported by their finding, and also that of Watt (1966), that in a depth profile of a water body P.E.R. values are highest at the surface where light inhibits photosynthesis and at the bottom of the euphotic zone where lack of light is inhibitory. Their warning is also supported by their finding that over a period of a year in the waters of a harbour, photosynthetic primary production varied from 1-32 $\mu gCl^{-1}hr^{-1}$ while extracellular release varied over the much narrower range of 0.5-2.0 $\mu gCl^{-1}hr^{-1}$.

Several authors believe that extracellular release may actually be underestimated by conventional techniques on the grounds that (i) bacteria utilise dissolved extracellular products almost as fast as they are released (Larsson and Hagström, 1979), (ii) that short term experiments do not allow sufficient time for metabolic end products to attain maximum labelling and thus excretion of high molecular weight compounds is underestimated (Wiebe and Smith, 1977), (Mague *et al*, 1980), and (iii) that "culture shock" may be a normal part of life in a well mixed water body as algae are circulated between areas of differing light intensity (Sharp, 1977).

In conclusion it appears that extracellular release is a significant, if not dominant source of dissolved organic carbon in natural water bodies.

(b) In the experimental part of this thesis, glycolic acid, or glycolate, was chosen as an example of a carbon substrate produced in natural waters by extracellular release, a choice which was brought about largely by the extent to which extracellular release of this substance has been documented and because some understanding of the mechanisms of its release exist.

For example, Hellebust (1965) incubated 22 species of unicellular marine algae in pure cultures with $^{14}CO_2$ and found extracellular release to account for from 3 to 25% of carbon fixed. Twenty-one species released glycolate of which three released greater than 10% of extracellular products as glycolate. Fogg (1971) believes that different culture conditions in Hellebust's experiments would have favoured greater release of glycolate.

Fogg (1977) reports that glycolate is often the major component of phytoplankton extracellular release and cites the work of Nalewajko, Chowdhuri and Fogg (1963) who found that glycolate is the only extracellular product of *Chlorella pyrenoidosa* in pure culture, short term experiments. Similar results were found by Bowes and Berry (1972) for *Chlamydomonas reinhardtii*. Watt (1966) showed by chromatography that between 13 and 92% of the ^{14}C-organic material released by three natural populations of phytoplankton was in the form of glycolate and Al-Hasan (1976) showed that 35% of the material released by a marine phytoplankton population was in the form of glycolate.

Tanaka, Makanishi and Kadota found by chromatography that glycolate was a major component of the extracellular release of a pelagic, marine phytoplankton population.

The mechanism of glycolate production in algae is as follows (Tolbert, 1974). As part of the carbon fixing Calvin cycle - Ribulose-1,5-bisphosphate (RuBP) has a CO_2 molecule added to it and the resulting six carbon molecule is immediately split to yield two molecules of 3-phosphoglycerate. This reaction runs in competition with another similar reaction in which the Ribulose-1,5-bisphosphate molecule is oxidised, by O_2, and immediately split to yield one molecule of 3-phosphoglycerate (3C) and one molecule of phosphoglycolate (2C). This latter compound is dephosphorylated to glycolate. At times, production of glycolate can result in an excessive build-up in the cell and as most algal types, except for blue green algae, have only a limited capacity to utilise glycolate, it is lost by diffusion through the cell

membrane. Intracellular utilisation mainly consists of the production of glycine and serine.

Whether or not glycolate loss is advantageous to algae is unclear. It has been suggested that it is an unavoidable result of the dual carboxylase/oxygenase function of the CO_2-fixing enzyme Ribulose-1,5-bisphosphate carboxylase, with excess glycolate formation occurring when CO_2 is limiting or O_2 is in excess. Alternatively, glycolate excretion may be a means by which photosynthesising cells, which are not energy-limited, dispose of excess reducing potential, in order to maintain an optimum redox state for the uptake of some limiting nutrient. "Slip" reactions of this type are discussed by Tempest and Neijssel (1978).

(c) One thing is clear from the physiology of glycolate production, and that is, that without the light-driven production of ATP, RuBP can not be formed and consequently, neither can glycolate. In other words, glycolate excretion at night is very unlikely.

Experimental evidence supports the hypothesis that in general the release of extracellular products by algae does not occur at night. Mague, Friberg, Hughes and Morris (1980) incubated a natural marine phytoplankton population with $NaH^{14}CO_3$ at *in situ* light levels and monitored the accumulation of extracellular products over 24 hours. Net extracellular products increased during daylight, remained constant overnight and continued increasing the next day. Saunders (1976) reported a similar experiment in which extracellular products increased during the day but decreased by 25% at night, presumably due to bacterial uptake. Eppley, Holmes and Paasche (1967) found that in cultures of *Ditylum* under artificial light/dark cycles, only small amounts (2-3%) of assimilated carbon were excreted in the dark, but sometimes larger amounts were excreted during the early hours of darkness.

(d) There is evidence that glucose occurs in natural waters, largely as a result of processes other than extracellular

release. Hellebust (1965) did not find glucose in the media of any of the 22 species of marine algae which he studied in unialgal culture. In 1974, in a review on extracellular products, Hellebust reported that simple sugars are usually found in small amounts in extracellular products of algae.

Glucose is probably present in natural waters, largely as the result of the slow and continuous breakdown of polysaccharides during the decomposition of dead algae and allochthonous carbon particles. This hypothesis is supported by the finding of Wright (1968) that the V_{max} for glucose uptake increased with depth in a lake while the V_{max} for glycolate decreased with depth. This finding suggested that the main source of glucose was the hypolimnion or sediments while the main source of glycolate was the photic zone. A similar trend of glucose V_{max} increasing with depth was reported by Overbeck (1975). Wood and Chua (1973) found that glucose concentration increased with depth and suggested that sediment decomposition was the source of most of the dissolved glucose in the lake in which they worked.

Evidence that glucose concentration in some aquatic ecosystems might be related to extracellular release is provided by Hanson and Snyder (1980) who reported a diurnal trend in glucose concentrations in a salt marsh estuary in which glucose concentration was maximal in the late afternoon and minimal at dawn.

If glucose <u>is</u> mainly a decomposition product then glucose supply is not likely to change on a diurnal basis. Thus, glucose availability will only change if demand for glucose changes on a diurnal basis. This might happen as a consequence of changing supply of another substrate, such as glycolate. A study of diurnal changes in glucose uptake kinetics might be expected to provide an interesting comparison with a similar study using glycolate as a substrate.

1.4 IS IT REASONABLE TO PROPOSE THAT CHANGING SUPPLY OF PDOC COMPOUNDS ON A DIURNAL BASIS COULD BE RESPONSIBLE FOR CHANGES IN UPTAKE KINETICS FOR PDOC COMPOUNDS BY AQUATIC HETEROTROPHIC MICRO-ORGANISMS?

To answer this question would require an understanding of the following two areas:

(1) The speed with which PDOC concentrations are known to change in response to changing supply and demand;

(2) The speed with which bacterial cells are capable of adapting substrate uptake systems.

To answer the above question with respect to a particular PDOC compound (glycolate in this case) would require a further understanding of:

(3) The role of glycolate in the nutrition of the heterotrophic population involved in its uptake;

(4) The role of glycolate as a growth limiting nutrient.

1.4.1 The Speed with which the Concentration of PDOC Compounds are Known to Change in Response to Changing Supply and Demand

In order for the heterotrophic bacteria in a lake to change their uptake kinetics for a substrate on a diurnal basis, they would require some form of stimulus. It is proposed that changing substrate concentration provides that stimulus. For this to be the case, demand for the substrate in question would have to be large enough to significantly deplete the substrate pool when supply is cut off at nightfall and depletion would have to be rapid enough so that for a part of the 24 hour cycle there is a lowered concentration of the substrate.

Literature values for the turnover times of PDOC as a whole are one hour (Larsson and Hagström, 1979) and ten hours (Wiebe and Smith, 1977) to several hundred hours (Bell, 1980), all being the results of studies on mixed substrates produced by algae from ^{14}C-DIC (dissolved inorganic carbon). Some of these results are within a range which would be sufficient to cause significant depletion of PDOC overnight.

Values for the turnover of glycolate, a known component of PDOC, are higher. Wright and Shah (1975) found turnover times of from 7-2500

hours in an estuarine environment with the range being due to season, sampling site and depth. Most values were greater than 24 hours. Wright (1968) measured turnover times of 60-200 hours in a lake and in another lake a turnover time of 300 hours was calculated from the data of Robinson, Hendzel and Gillespie (1973) using a respiration correction factor from Wright (1968). In most, but by no means all, of these cases the turnover times are too long to permit glycolate depletion within a diurnal cycle and under the circumstances that most of these times were measured, changes in glycolate uptake kinetics would not be expected to occur. However, the possibility can not be discounted that glycolate turnover times are much shorter in other waters. The turnover of glucose, for which much more data exists, ranges over four orders of magnitude from 15 minutes to 10,000 hours, in a variety of water bodies (Hobbie and Rublee, 1977). Faster turnover is frequently associated with eutrophic waters.

Direct evidence that the concentration of PDOC changes on a diurnal basis is provided by the following authors. Gocke (1970) reported an increase in the concentration of free amino acids and dissolved peptides from 150 μgl^{-1} to 200 μgl^{-1} during the day followed by a decrease to 150 μgl^{-1} at night in the more productive of the two lakes which he was studying. Meyer-Reil *et al* (1979) found changes in the concentrations of amino acids, glucose and fructose over one 24 hour period, at three sites in the Baltic Sea. Although amino acids are known to be algal extracellular products (Gocke, 1970), the same can probably not be said of glucose (see section 1.3(d)). Whether or not the observed concentration changes were diurnally cyclic was not apparent. Walsh (1965b) reported an increase in dissolved carbohydrate from about 1,500 μgl^{-1} to 3,000 μgl^{-1} during daylight hours followed by a decrease at night on the two occasions on which dissolved carbohydrate was measured over a 24 hour period in a highly eutrophic saline pond.

In conclusion, previous workers have shown that the concentrations of some PDOC compounds do have the potential for rapid enough change within a 24 hour cycle to act as a possible stimulus for the adjustment of substrate systems by aquatic heterotrophs. However, they have also shown that such rapid change is not likely to occur for all substrates, at all times in all lakes.

1.4.2 The Speed with which Bacterial Cells are Capable of Adapting Their Substrate Uptake Systems

Adaptions could be either phenotypic or genotypic or both.

(a) Genotypic Changes

As uptake kinetics can only be measured for a population as a whole, changes could be due to changed species dominance, each species having different uptake kinetics. There are a number of examples of changing species dominance in pseudo-natural environments, such as chemostats. Matin and Veldkamp (1978) reported that in a mixed, lactose-limited chemostat culture a species of *Spirillum* dominated over a *Pseudomonas* sp. at low lactate concentration whereas the *Pseudomonas* dominated at high lactate concentrations. The reason given was that the *Spirillum* sp. had a three-fold lower rate constant for transport of lactate than the *Pseudomonas* sp. and could take up lactate faster at the low concentration, and at the high concentrations the greater maximum activity of the lactose-utilising enzymes of the *Pseudomonas* sp. gave it a two-fold greater maximum growth rate than the *Spirillum* sp.

Slater and Godwin (1980) is a recent review of changing species dominance in laboratory cultures.

The major objection to a theory of a succession of dominant species over a 24 hour cycle arises from the very slow growth rates found in natural waters. Reported values of doubling times are 20-40 hours, with a minimum of eight hours, for the actively growing part of an estuarine microbial population (Meyer-Reil, 1977), 2 - 10 hours for bacterial microcolonies growing on a glass slide in a freshwater pool (Brock, 1971) and generally much longer times for the doubling times of populations as a whole, e.g. ten to 280 hours (Brock, 1971), 17-600 hours (Kuznetsov, 1977), 50-100 hours (Jannasch, 1979) and 10-100 hours (Hagström *et al*, 1979). Williams and Gray (1970) found that an initial lag of 20-30 hours before amino acid oxidation increased in amino acid enriched seawater, an effect which was put down to population growth.

The doubling times of whole aquatic microbial populations leads one to the conclusion that changing species dominance is unlikely to be important on a diurnal basis. This is also the conclusion from the growth rates of some sub-populations, e.g. Williams and Gray (1970). However, a few subpopulations have been shown to grow fast enough to have the potential to cause dominance changes, and presumably kinetic changes on a diurnal basis, e.g. Brock (1971) and Meyer-Reil (1979), above. Whether the scarcity of examples of rapid growth of bacteria in natural waters is a reflection of the actual situation in natural waters or whether it is due to a lack of research into this area, can not be determined at present.

(b) Phenotypic Changes

Phenotypic changes are well documented and include:

(i) The induction and repression of single enzyme systems.

In a review of chemostat studies of microbial regulation in the face of nutrient depletion, Matin (1978) found that the most common strategy, as far as induction/repression is concerned, is for bacteria to increase synthesis of enzymes of catabolism of a limiting carbon substrate as that substrate becomes depleted. This was explained as a reduction in catabolite repression, implying that conditions were not very impoverished to start with. In natural waters the two other strategies, a decrease in enzyme production with decreasing substrate concentration, or maximal enzyme production at an intermediate substrate concentration might be more common. These strategies would fit in with the theory of Stevenson (1978) that most bacteria in natural waters are in a dormant, repressed state due to nutrient depletion, and also with the experimental findings of Vaccaro and Jannasch (1967) that a marine microbial population could not actively transport amino acids immediately upon their addition to seawater.

The induction/repression of single enzyme systems for a substrate such as glycolate would involve changes in the V_{max} for glycolate uptake with no corresponding changes in K_T.

(ii) The induction and repression of multiple enzyme systems for the same substrate.

For example, *Klebsiella aerogenies* has two uptake pathways for glycerol. The first, a low affinity ($K_T = 4 \times 10^{-2} gl^{-1}$), high V_{max} system involving glycerol dehydrogenase is used at high glycerol concentrations. The second involving glycerol kinase has a higher affinity ($K_T = 2 \times 10^{-6} gl^{-1}$) and lower V_{max} and is used at low concentrations (Neijssel *et al*, 1975).

Matin (1978) reported that the lactate dehydrogenase activity of a *Spirillum* sp. has three distinct pH peaks with the peak heights changing relative to each other depending on lactate availability.

A multi-enzyme system could also include different enzymes in different species (c.f. Matin and Veldkamp (1978) in section 1.4.2(i) of this thesis.

If different enzymes were induced or repressed at different times of the day in response to changing glycolate levels the expected change in kinetics would be a high V_{max} and high K_T during the day and a low V_{max} and low K_T during the night.

Any form of induction/repression mechanism would depend on, firstly the speed of induction and secondly the half lives of already manufactured enzymes, i.e. if induction could not occur fast enough or if enzymes remained stable for long periods after repression, changes in kinetics would not be seen on a diurnal basis.

(iii) Induction.

Induction times of laboratory cultures are certainly short enough to result in changes in uptake kinetics on a diurnal basis. Koch (1971) reports that when *E.coli* grown in a glucose-limited chemostat at a doubling time of eleven hours was "shifted up" to what would ultimately give a doubling time of 40 minutes, protein synthesis increased seven-fold during the following 40 minutes. If this additional protein synthesis was due solely to the metabolic capacity of already existing enzymes, the increase in protein synthesis would have been immediate. *E.coli* can also synthesise β-galactosidase within minutes of induction by lactose analogues, even at doubling times as low as 24 hours (Koch, 1971). Reports on response times in natural waters are less clear cut. Induction of active uptake systems for a variety of carbon substrates occurred in less than 20 hours when seawater samples were enriched with the substrate in question (Vaccaro, 1969). Williams and Gray (1970) found that in seawater enriched with amino acids there was an immediate increase in absolute amino acid oxidation rate above that found in unenriched waters. It was not clear from the paper of Williams and Gray whether this was an effect of induction or purely the effect that increased amino acid concentration would have on oxidation rates with already existing enzymes.

(iv) Enzyme Degradation after Repression.

Protein turnover is an important part of the regulatory processes of mammalian cells (Schimke, 1975) (Goldberg and St.John, 1976). From work on mammalian cells it is clear that certain key enzymes in metabolic pathways have short half lives in the order of .1-3 hours. This is to enable metabolic pathways which are no longer required and, which may in fact

be deleterious under new environmental conditions (e.g. biosynthetic pathways under nutrient limiting conditions), to be shut down rapidly after enzyme repression. The situation in bacteria is less clear, but probably similar. Pine (1973) showed that most proteins in *E.coli* have half lives in the order of 25-50 hours. From 1 to 7% have half lives of 10-30 minutes, of which none of those isolated is specifically involved in transport.

Whether or not permeases are important enough to warrant the metabolic expense of short half lives is not clear. For any given species this would presumably depend mainly on (a) whether individuals are exposed to rapid and frequent changes in the dominant carbon substrate species, and (b) whether or not they have sufficient membrane area to synthesise new permeases without first removing old ones. Both of these factors are unknown. However, as pointed out by Krambeck (1979) uptake kinetics, as measured by the techniques of Parsons and Strickland (1961) are not necessarily the kinetics of a permease system alone. They could be the kinetics of the entire metabolic pathway for the substrate concerned. If intracellular utilisation of the substrate is prevented by degradation of a key enzyme, then so is measured accumulation.

To return to the original question, it appears that, in theory at least, bacteria have the potential to induce, repress and inactivate the enzymes responsible for uptake kinetics and on short enough time scales to enable changes in kinetics to be observed on a diurnal basis.

1.4.3 The Nutritional Role of Glycolate

As glycolate is a reduced carbon compound, it would be reasonable to suppose that, in aerobic waters, it could act as both a carbon and an energy source for heterotrophic bacteria. However, there is some inconclusive evidence that this is not the case. Wright and Shah (1975) reported that, of 141 colonies from coastal seawater, 63% could

oxidise ^{14}C-glycolate but none out of 84 colonies tested could grow in a medium containing glycolate as the sole carbon and energy source. However, seven of the 16 cultures tested could achieve greater population densities in media containing other carbon compounds, such as lactate, glucose or acetate, if those media were supplemented with glycolate. Wright and Shah suggested that glycolate is used only as an energy source by most aquatic bacteria. They used, to support this suggestion, the finding that about 80% of the glycolate taken up in their heterotrophic uptake experiments was oxidised and the finding that diluting natural seawater samples with artificial seawater enriched with glycolate yielded growth down to a 10^{-1} dilution (0.1 ml sample in 1.0 ml medium) whereas dilution with artificial seawater enriched with glucose, lactate or acetate at the same concentration, yielded growth down to 10^{-5} dilution.

In the context of the present discussion, the importance of their finding is that, as they suggest, variations in community heterotrophic ability to utilise glycolate may not be caused by glycolate availability but primarily by the availability of other carbon sources which may or may not fluctuate in supply on a diurnal basis.

However, Wright (1968) and Konings and Veldkamp (1980) have both isolated aquatic bacteria capable of growing on glycolate as a sole carbon/energy source. The importance of species able to utilise glycolate as sole carbon/energy source relative to other species in the heterotrophic communities from which they were taken was not ascertained.

Without resolving this issue it is still possible to examine the possible effects of fluctuating glycolate concentrations on aquatic heterotrophic bacteria, in terms of whether glycolate (as a carbon source or an energy source or both) is or is not a growth limiting nutrient.

1.4.4 The Role of Glycolate as a Growth-Limiting Nutrient

There is some indirect evidence that carbon and/or energy sources, by their low availability, provide one of the major limitations to the unhindered growth of pelagic, heterotrophic bacteria. This evidence is, as suggested by Stráskrabová and Komárkorva (1979), the annual relationship between bacterial biomass and phytoplankton (primary producer) biomass in water bodies. Peaks in bacterial numbers often

coincide with, or lag slightly behind, peaks in phytoplankton biomass. As it would be extremely unwise to extrapolate such a general conclusion to cover the hour-by-hour existence of a bacterial population, it will be necessary to look at each of the three possible relationships between glycolate supply and nutrient limitation, and to discuss each relationship in terms of its possible effect on diurnal changes in glycolate uptake kinetics. Over each 24 hour period the possibilities are:

(a) The Supply of Glycolate is Never Growth-Limiting

There is evidence to show that when a substrate serving as a carbon/energy source is available at non-limiting concentrations, bacteria do not regulate metabolism of that substrate to give a maximum growth yield on that substrate. Rather, they tend to overutilise it in order to produce the reducing potential, transmembrane potential and metabolic intermediates required to hasten uptake of whichever nutrient happens to be growth limiting (see review of Tempest and Neijssel (1978)). While wastage of carbon/energy in the "slip" reactions reported by Tempest and Neijssel obviously yields a growth, and hence competitive, advantage to bacteria which adopt this strategy, there is no evidence to suggest that maximum possible wastage is advantageous. In fact, Tempest and Neijssel present results which show that under conditions of carbon/energy excess *Klebsiella aerogenes* utilises carbon/energy sources at rates dependant on exactly which other nutrient is limiting (Tempest and Neijssel, p.120). In terms of the heterotrophic uptake technique this means that different uptake kinetics could be expressed for a given carbon/energy substrate by a population which is always supplied with an excess of carbon/energy. However, with regard to the short term changes in supply of a given substrate (e.g. glycolate), it is not possible to say whether regulation of the uptake and metabolism of that substrate is likely to occur in order to give optimum "slip" when that substrate is present in excess as a carbon/energy source.

(b) Glycolate, along with other carbon/energy substrates, may be Growth Limiting over the whole 24 Hour Period

Once again there is evidence that cells limited by a carbon/energy substrate adjust their capacity to metabolise that substrate as its concentration changes. Matin (1980) found that in 51 reports on the influence of chemostat dilution rate on the enzyme synthesis of carbon/energy limited cells, only three referred to situations in which enzyme synthesis did not change. In the remaining 48 instances, the direction of change varied, with some reports showing increasing cellular enzyme synthesis in response to limiting substrate, others showing a decrease and yet others showing maximum activity of the culture at an intermediate substrate concentration.

(c) Glycolate may be Limiting for Part of the Day (presumably predawn) and in Excess for the Rest (late afternoon)

Lacking information on changes of the steady state metabolic capabilities of cells which have been switched from one nutrient limitation to another. I venture to suggest that such changes occur and that the directions are as diverse as in (b) above.

In summary, although the role of glycolate as a growth-limiting nutrient has not been established, previous work suggests that aquatic heterotrophic populations might alter their ability to take up glycolate in response to changing glycolate concentration whether glycolate is in excess or is a limiting nutrient and even if the changing concentration of glycolate does not change the relationship between glycolate supply and nutrient limitation.

1.4.5 The Question which Prompted the Preceding Discussion Was: Is it Reasonable to Propose that Changing Supply of PDOC Compounds on a Diurnal Basis could be Responsible for Changes in Uptake Kinetics for PDOC Compounds by Aquatic Heterotrophic Micro-Organisms?

On the basis of what is known about (1) the speed with which PDOC concentrations change in response to changing supply and demand, (2) the speed with which bacterial cells are capable of adapting their substrate uptake systems, (3) the role of glycolate as a nutrient

and (4) the role of glycolate as a growth-limiting nutrient, the answer to the above question is "yes". It is also "yes" if glycolate is substituted for PDOC compounds in general. More correctly there is no evidence to suggest that the proposal in question is an unreasonable proposal either in the general or the specific case.

However, even if the above proposal is reasonable there is evidence that changing substrate concentrations will not always stimulate changes in the kinetics of substrate uptake. There is also no certainty that glycolate concentrations in lakes will always change as a result of the changing balance between extracellular release and heterotrophic uptake which is assumed to occur diurnally. An examination of glycolate uptake kinetics on a diurnal basis would appear to be worthwhile but, in view of the above uncertainties, changes in the uptake kinetics are not certain to occur.

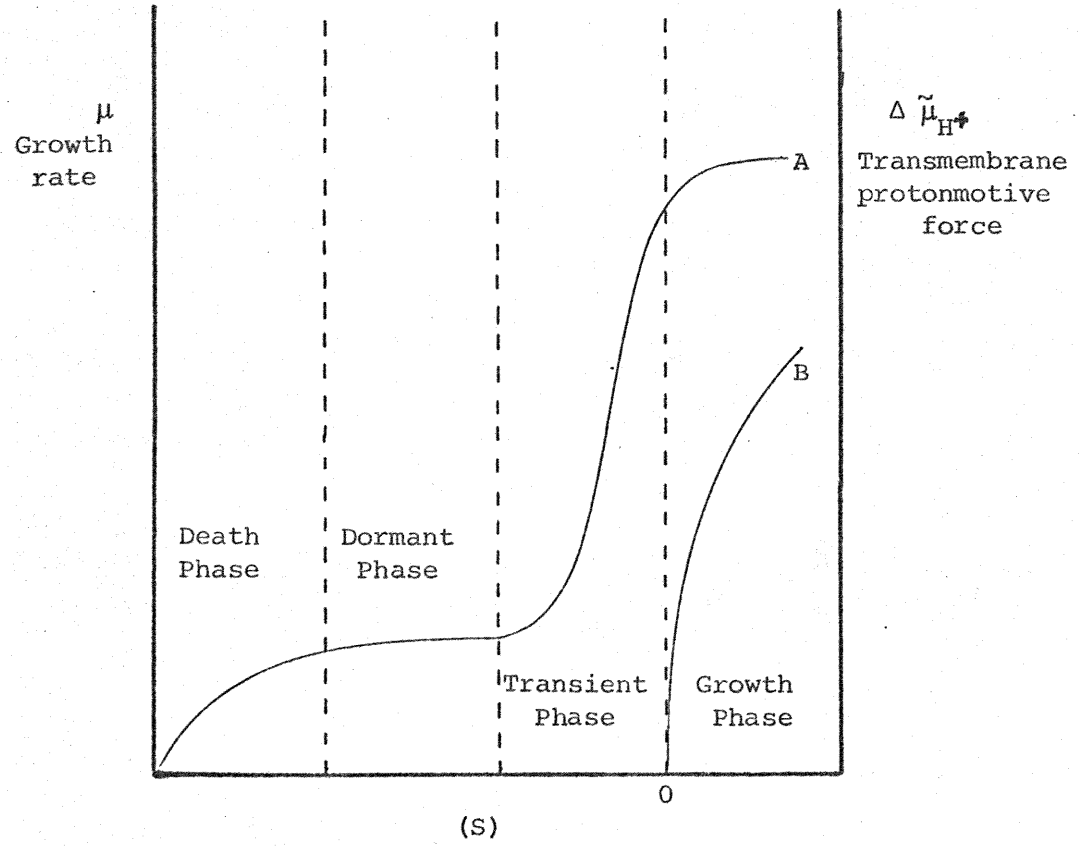


Figure 1.5/1 - Decrease of the specific growth rate (μ) of an energy-limited cell to zero (B) and the hypothetical change of protonmotive force in various phases (A). Substrate (S) is the energy source. (Konings and Veldkamp, 1980)

1.5 TO WHAT EXTENT CAN A DIURNAL STUDY OF HETEROTROPHIC UPTAKE KINETICS BE USED TO IDENTIFY THE STRATEGIES WHICH AQUATIC MICROHETEROTROPHS ARE ADOPTING IN RESPONSE TO CHANGING NUTRIENT LIMITATION?

The strategies adopted by bacteria in response to changing nutrient limitation can be conveniently discussed in terms of the following general hypothetical model for a bacterium shifting from growth under energy limitation via dormancy to death. This model was produced by Konings and Veldkamp (1980). In it transmembrane proton-motive force decreases in a series of discrete stages after the energy supply runs out (see figure 1.5/1).

When the energy supply is gradually decreased the specific growth rate (μ) decreases. Initially, the organism can maintain the proton-motive force at a high level, but below certain levels of energy supply the proton pump activity decreases and consequently the proton-motive force also decreases. Gradually, as endogenous and exogenous substrate dwindle, the cell reaches a stage where it can just maintain sufficient proton-motive force to retain cellular components essential for viability. In this, the dormant stage, energy-consuming functions such as ATP synthesis, biosynthesis, nitrogen fixation and mobility, among others, have stopped. If remaining energy consumption can be kept to a minimum, viability can be retained for long periods. Eventually, however, endogenous reserves become depleted and, if new utilisable energy sources do not become available, the remaining proton-motive force is dissipated and biosynthesis can no longer replace essential cellular constituents which are lost by the cell's normal degradative processes or by increased diffusion across the now unpolarised membrane. Loss of viability ensues. For any individual cell, the length of time each particular phase can last and the concentration range over which the transition phase occurs will depend upon the genetic capability of that cell and its previous growth conditions.

It should be emphasised that the above account is only a model; an equally applicable model might utilise the adenylate charge concept of Atkinson and Walton (1967) in which energy charge can be substituted for the proton-motive force in the model of Konings and Veldkamp. (See the review of Knowles, 1977).

From the point of view of discussing adaptations of the bacterial cell to growth limiting conditions it is necessary to consider mechanisms adopted by bacteria finding themselves in the 'transient phase' of the model proposed by Konings and Veldkamp.

These mechanisms may include:

(i) The simultaneous utilisation of a variety of substrates (Tempest and Neijssel, 1978).

(ii) The induction of high-affinity uptake systems for the limiting carbon/energy substrate (Dawes, 1976).

(iii) A change in cell morphology to give a greater surface area to volume ratio (Poindexter, 1979).

(iv) The endogenous metabolism of intracellular storage materials, such as glycogen, carbohydrates and poly-β-hydroxybutyrate and the utilisation of non-essential cellular components such as some proteins, as energy sources (Dawes, 1976).

If these first four mechanisms fail and dormancy ensues, a further mechanism may be:

(v) The adoption of specialised survival and dispersal forms in which the dormant state can be maintained for long periods of time. Spores are a good example of this (Ellar, 1978).

Use of one or more of these above mechanisms by bacteria under growth-limiting conditions enables us to classify them in very general terms as either k-strategists or r-strategists.

In simple terms the k-strategist attempts to prolong the transition phase for as long as possible. It is characterised by a low maintenance energy requirement, the ability to metabolise a diverse variety of compounds, the ability to alter surface area to volume ratio, by storage polymers, high-affinity uptake systems, and as a consequence of the need to maintain a diverse metabolic machinery at any one time, for the simultaneous uptake of many carbon/energy substrates, it is unable to achieve high rates of growth relative to an r-strategist. Thus, it can be seen to use (i), (ii), (iii) and (iv) of the above mechanisms.

The r-strategist specialises in the metabolism of a narrow range of substrates and when these substrates are abundant it can achieve high

growth rates. When its particular substrates have been depleted the r-strategist does not try to maintain maximum metabolic activity. It tends to move immediately to a dormant stage in which it waits for conditions suited to its high growth capability. Dispersal forms are a characteristic of some r-strategists, spores being a prime example. The r-strategist is also characterised by a high maintenance energy during active growth. This causes it to switch off metabolism and enter a dormant phase at a substrate concentration at which k-strategists are actively growing. Thus, r-strategists utilise predominantly (iv) and (v) of the above techniques.

Koch (1978) suggests that all bacteria exhibit strategies characteristic of both k-strategists and r-strategists, the exact nature of the response by a given species of bacteria to a fluctuating nutritional environment being dependent on the selective pressures of the fluctuations in the environment to which it is accustomed. For example, *E.coli*, when grown aerobically on glucose at a concentration of 1,500 μgl^{-1}, and then allowed to deplete the glucose in its medium, will respond with maximum glucose uptake and growth yields within two minutes of the glucose concentration having been returned to 1,500 μgl^{-1}, if starvation has lasted for only one hour (Koch, 1978). After 12, 24 or 48 hours of starvation maximum uptake still occurs within minutes of supplementation with glucose but growth yields take progressively longer to reach their maximum levels; from several minutes after 12 hours starvation up to several hours after 48 hours starvation. If these results can be applied to the natural environment of *E.coli*, the mammalian gut, then in this environment *E.coli* is capable of maintaining full activity for as long as the normal lengths of time to which it would be exposed to nutrient depletion, i.e. fluctuations could occur from minute to minute in response to mixing of gut contents, or every day in response to feeding of the host. Thus, in the gut *E.coli* is a k-strategist. However, *E.coli* is capable of survival for many days outside the gut, in soil and water, a fact of significance to its dispersal to new hosts and also of public health significance (Vasconcelos and Swartz, 1976). From Koch's work it can be inferred that survival in the relatively impoverished environment outside the gut involves a state of reduced metabolic potential or semi-dormancy. Full metabolic potential is only achieved <u>after</u> a period of exposure to a new nutrient supply. In this context *E.coli* is an r-strategist.

Thus it can be seen that classification of a particular bacterium as either an r-strategist or a k-strategist must be made with reference to a particular set of fluctuations, with the length of time between periods of nutrient sufficiency and the regularity of fluctuation being particularly important. Another important reference is the set of strategies adopted by different bacterial species co-existing in the same fluctuating environment.

The question giving rise to this discussion could be re-phrased as follows. Would it be possible to attribute any given diurnal pattern of kinetic change to one of the two strategies, r or k? These strategies are with respect to a diurnal cycle of substrate supply and the k and r terms are used here to label trends in either direction on a continuum of possible strategy.

If it is assumed (i) that in the water body under consideration, the concentration of the carbon/energy substrate being investigated increases during the day and decreases at night, and (ii) that the measured kinetics are attributable to one strain of identical bacteria, then the following table illustrates which strategies would be expected to give rise to each of the simple combinations of K_T and V_{max} increasing, decreasing or not changing at night. In each case the opposite direction of change is assumed to occur during the day time.

	V_{max}		
K_T	increase	decrease	no change
increase	?	?	?
decrease	k	k	k
no change	k	r	k/r

The question marks indicate that neither strategy is likely to involve the induction of a low-affinity (high K_T) uptake system in response to a decreasing substrate concentration. Any decrease in K_T would involve induction of a high-affinity uptake system in response to decreasing substrate; a technique typical of the k-strategy. Similarly, maintaining uptake rate by increasing the number of molecules of an already existing permease is an example of k-strategy (K_T not changing, V_{max} increasing). A decrease in V_{max} with no change in K_T indicates

metabolic "switching off", indicative of an r-strategy. No change in either parameter indicates insufficient stimulus for change in the decreasing substrate concentration; either strategy.

In practice, the following factors will tend to decrease the usefulness of the above generalisations:

(i) Without measuring the natural substrate concentrations (S_n) at each time of day the value of K_T can not be calculated from the measured parameter ($K_T + S_n$).

(ii) The measurement of S_n is also necessary if the assumption that the substrate concentration is lower at night is to be validated.

(iii) No consideration has been taken above of the effect of 'preferential' substrates, which, by themselves fluctuating in concentration, may cause the observed patterns of change in the uptake kinetics of the substrate in question. This would result in serious misinterpretation only if the pattern of change of the 'preferential' substrate was opposite to that of the assumed pattern of change of the studied substrate.

(iv) A pattern of change of uptake kinetics which was not diurnally cyclic or was cyclic but not unimodal would be difficult or impossible to interpret. The supply of carbon/energy substrates in lakes may involve fluctuations with a variety of repeat periods. By overshadowing the proposed 24 hour cycle of substrate supply, any one of these fluctuations might be responsible for non-cyclic or multimodal patterns of kinetic change on a diurnal basis.

(v) In practice, the measured kinetics result from the combined or 'average' kinetics of many different subpopulations of bacteria which may adopt quite different strategies, resulting in different, possibly opposing, patterns of kinetic change and consequently an overall pattern of kinetic change which might not reflect that expected from either strategy.

Factors (i) and (ii) above are technical problems, which may or may not be surmountable. Factor (iii) is surmountable only by assuming that, in the absence of a known mechanism to cause such an increase, a diurnal increase in the supply of a carbon/energy substrate at night is unlikely to occur. Factor (iv) is outside the control of the experimenter and cannot be predicted in advance. Furthermore, it might not

be unreasonable to suppose that such fluctuations in carbon/energy supply may vary seasonally and from place to place. Factor (v) almost totally undermines the value of the heterotrophic uptake technique for examining bacterial strategy in the manner described. It can reasonably be assumed that subpopulations adopting each strategy (k or r), and a mixture of strategies intermediate between the two extremes, will be found in any water body. Assessing the relative importance of each (in terms of biomass, growth increment or substrate uptake etc.) to the population as a whole, would be impossible from 'averaged' population results.

Chapter 2

MATERIALS AND METHODS

2.1 INTRODUCTION

In the introduction to the literature review (section 1.1), it was stated that the aim of this thesis was to determine whether aquatic heterotrophic bacteria modify their ability to take up certain substrates, on a diurnal basis, in response to diurnal changes in the extracellular release of those substrates by algae. To demonstrate modified uptake ability of a given heterotrophic population it would be sufficient to determine the heterotrophic uptake kinetics of that population over one, or preferably several, 24 hour periods, whilst simultaneously ensuring that the same population, or its progeny, was being examined over any 24 hour cycle. That is, it would be necessary to ensure that population changes were not due to mixing water masses or migration. The former problem was experienced by Gocke (1975) who looked at the uptake kinetics of glucose and acetate at four to six hour intervals over three days as part of a study of short-term variations in microbiological and chemical parameters in Kiel Fjord, West Germany. Random fluctuations were found, which considered along with other chemical, physical and biological data led Gocke to suggest that the mixing of heterogeneous water masses was responsible for much of this variation.

The latter problem, migration, could only involve zooplankton and phytoplankton. The former can be excluded by coarse filtration through nylon netting. Phytoplankton can not be excluded from water samples without also excluding some bacteria as the size range of phytoplankton overlaps that of detrital particles to which some aquatic bacteria adhere. Phytoplankton migration on a diurnal basis is well documented (see, for example, Tilzer (1973)).

Migration could have two effects on the interpretation of results in this investigation. (a) Diurnal changes in extracellular release at a certain depth due to algal migration into and out of that depth may not be distinguishable from changes in extracellular release due to physiological changes within the algae themselves. (b) If algae are responsible for at least some of the uptake of organic carbon compounds in natural waters then any observed changes in uptake kinetics may be caused by migration rather than population adaption. It has not been unequivocally shown that algal uptake at naturally occurring substrate concentrations plays an insignificant role in the determination of

heterotrophic uptake kinetics although this is believed to be the case by Wright and Hobbie (1965, 1966). Other authors, including Allen (1971) and Vincent and Goldman (1980) have produced evidence that algal uptake may be significant, especially uptake by very small species (nanno-plankton) with surface area to volume ratios approaching those of bacteria and by non-motile algae in waters in which the depth of turbulent mixing exceeds the depth of the photic zone. However, in the case of glycolate as a substrate, Tolbert (1974) concludes that "it is unlikely that much of the glycolate excreted in natural waters is re-utilised by algae".

In this investigation, total bacterial numbers were determined using epifluorescent microscopy and algal biomass was monitored by chlorophyll estimation. It was hoped that these two parameters would indicate only gross changes due to mixing or migration. In Lake Rotomanuka all water samples were also filtered through a 100 μm plankton net prior to use to remove larger zooplankton. This technique was not used in Lake Fryxell in which large zooplankton are not found.

The second aim of this thesis was to investigate the strategies which aquatic bacteria adopt in response to diurnal changes in substrates which serve as sources of carbon or energy.

There are two major problems associated with this type of investigation (see section 1.5).

(a) Without measuring the natural concentration of the substrate, the uptake kinetics of which are being determined, it is not possible to calculate the value of the uptake rate constant, K_T, from the term $(K_T + S_n)$. (See section 1.2.3.) Thus, the only parameter available from heterotrophic uptake data which can be used to make inferences on strategy is V_{max}, which, as discussed in section 1.5 is of limited use on its own. An attempt was made to develop a technique to measure *in situ* glycolate levels but intitial problems with this technique resulted in only a limited amount of information on glycolate concentration being available.

(b) Aquatic heterotrophic populations can be assumed to be composed of many subpopulations each of which may respond to changing glycolate levels with a different strategy, thus making interpretation of kinetic data difficult. Two ways could be seen to partly remedy this situation. The first is to identify each subpopulation and its uptake at each concentration used in the heterotrophic uptake technique. This can be achieved with some degree of success by using grain density autoradiography to classify subpopulations according to uptake and morphology, as was demonstrated by Stanley and Staley (1975). However, the very high concentrations of substrate required for successful autoradiography (see Brock and Brock (1968)) would probably invalidate this technique in any aquatic environment except for a highly eutrophic one such as the oxidation pond studied by Stanley and Staley. There are also problems associated with the interpretation of grain density autoradiography (Knoechel, 1976). In view of these difficulties, this technique was not used.

A more simple method of dividing cells into subpopulations involves classification based on the criterion of whether or not cells have an active dehydrogenase system. This may be determined using the INT reduction/epifluorescent microscopy technique of Zimmerman, Iturriaga and Becker (1978). An assumption has to be made that cells which show no dehydrogenase activity are metabolically inactive and hence could not be responsible for significant substrate uptake. Thus two subpopulations may be detected, (i) those which <u>are not</u> responsible for uptake and (ii) those which <u>could be</u> responsible; dehydrogenase activity does not necessarily imply uptake of the substrate in question. However, this approach was also abandoned for methodological reasons which will be covered in section 2.7.

Another way of approaching the problem of the effect of a heterogeneous population on interpretation of diurnal changes in uptake kinetics is to examine a single bacterial species, isolated from other aquatic populations in a lake, but in

contact with the same water, in which levels of extracellular products change on a diurnal basis, i.e. in continuous culture using lake water as a medium or in cage culture.

This approach was considered but the values reported by Vasconcelos and Swartz (1976) for diffusion rates into a cage via walls composed of membrane filters, were too low for a cage to be useful for studying changes in water composition on less than a diurnal basis. Vasconcelos and Swartz were interested in long-term survival of coliform bacteria in seawater. To increase flow rates through a cage by pressurisation would have been impractical. Initial experiments showed that the Nuclepore polycarbonate filters recommended by Vasconcelos and Swartz clog very rapidly, when used for filtering even tap water. With vigorous stirring at the filter surface and a constant positive pressure, flow through a 0.1 μm filter decreased by 75% in 12 hours. Thus, the idea of using cage cultures to study individual species of bacteria in their natural environment was also abandoned.

With no method for distinguishing between the uptake kinetics of subpopulations or strains of bacteria and with only limited measurements of natural substrate concentration, it can be concluded that the results obtained would have little value as indicators of bacterial strategies adopted in response to fluctuating substrate availability (see section 1.5).

The methodology of the investigation consists primarily of the heterotrophic uptake techniques, including some glycolate measurements, bacterial direct counts and chlorophyll extraction. Minor techniques include the use of temperature and oxygen profiles of lakes to give some indication of the extent to which they are mixed.

Diurnal changes in heterotrophic uptake kinetics were studied in two lakes, Lake Fryxell in Southern Victoria Land, Antarctica and Lake Rotomanuka in the Waikato region of New Zealand. These lakes are described in sections 3.2 and 4.2. In Lake Fryxell a modification of the electron transport system activity (ETS activity) technique was

used in an attempt to determine where peaks of bacterial activity occurred in the water column and whether there was any migration of these peaks.

The techniques used are discussed more fully in the following sections.

2.2 HETEROTROPHIC UPTAKE METHODOLOGY

Parsons and Strickland (1961) were by no means the first to use ^{14}C-labelled organic compounds to study the metabolism of aquatic micro-organisms. However, their discovery that the relationship between substrate incorporation into biomass and substrate concentration could be described by the Michaelis-Menten equation was of major importance. By measuring substrate uptake rates at a range of added substrate concentrations they could produce three quantitative uptake parameters

(a) V_{max}, the maximum attainable value for uptake rate, which occurs at saturating substrate concentrations,

(b) T_t, the turnover time of the naturally occurring substrate,

(c) $(K_T + S_n)$, the sum of the uptake rate constant and the naturally occurring substrate concentration, from which maximum possible values for K_T and S_n can be obtained.

The findings of Parsons and Strickland encouraged extensive use of ^{14}C-labelled compounds in aquatic environments, which in turn was responsible for a wealth of literature on the methodology of the heterotrophic uptake technique, as the method of Parsons and Strickland was known. In the following sections, methodology and technical problems are discussed in terms of the methods which I used to measure bacterial uptake of ^{14}C-labelled substrates over a range of added substrate concentrations, in natural water samples. These methods are summarised in the following series of steps which coincide with the numbered sequence in figure 2.2/1

(1) Bulk water sample collected and filtered through 100 µm plankton net (sampling method different in Lake Fryxell; see section 4.2),

(2) 10 ml subsamples distributed into 20 ml syringes,

(3) ^{14}C-substrate added and syringes sealed airtight,

(4) Incubation,

(5) CO_2 trapping mixture applied to cylindrical filter paper supports,

(6) Sample acidified and flushed to drive off CO_2 which is trapped in filter paper supports by trapping mixture,

(7) Sample filtered to retrieve cells. Filters washed with 'cold' substrate,

(8) Filter paper supports and filters stored in sealed vials,

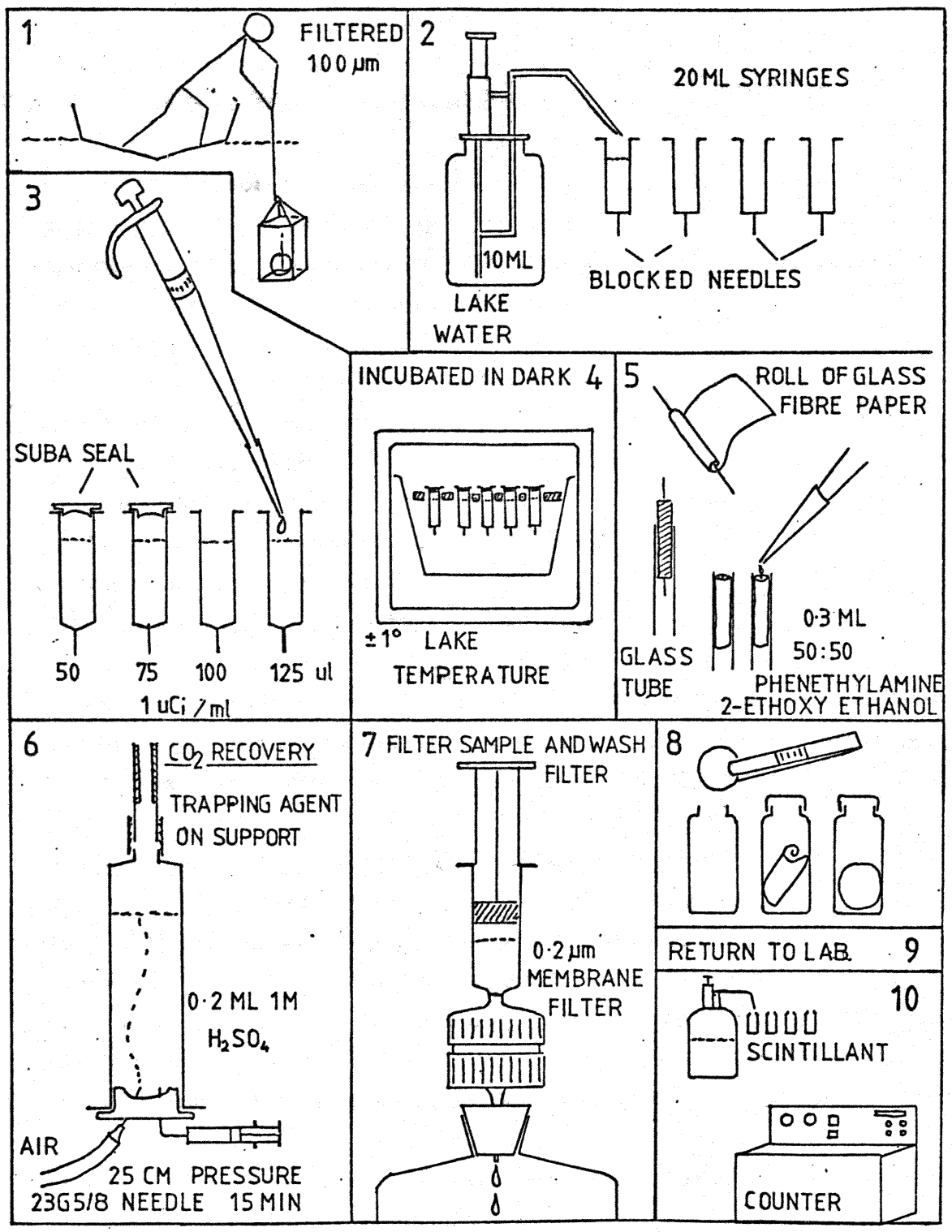


Figure 2.2/1 - A Diagrammatic Summary -- Heterotrophic Uptake Methodology

See text (section 2.2) for explanation

(9),(10) $^{14}CO_2$ on filter paper supports and ^{14}C on membrane filters counted by liquid scintillation to determine, respectively, the amount of substrate respired and the amount of substrate incorporated into biomass.

Rather than divorcing the experimental rationale from a more detailed description of the methodology it would seem more pertinent to discuss the development of each aspect of the methodology as it is described in more detail in the following sections:

1. Substrate preparation, dilution and storage.
2. Substrate addition and incubation duration and conditions.
3. CO_2 displacement and trapping.
4. Sample filtration.
5. Blanks and controls.
6. Liquid scintillation counting.
7. Decontamination and waste disposal.
8. Interpretation of results and determination of confidence limits.

An extensive coverage of the field of heterotrophic uptake methodology can be found in the reviews of Wright (1971), Thompson and Hamilton (1974) and Hobbie and Rublee (1977).

2.2.1 Substrate Preparation, Dilution and Storage

The two ^{14}C-labelled substrates used in this investigation were Na-[1-^{14}C]-glycolate and D-[U-^{14}C]-glucose, both obtained from the Radiochemical Centre, Amersham, England. Glycolate, as discussed in section 1.3, is an example of a compound excreted by algae. Glucose was chosen as a compound which does not appear to be excreted, to any great extent, by algae (section 1.3(d)).

Organic impurities in both ^{14}C-labelled substrates were assumed to be non-existent and the manufacturer's values for specific activity were accepted as the correct values. $^{14}CO_2$ was an impurity which presented some problems in the measurement of respired substrate. This could be overcome by the following technique. After dilution of stock substrate to provide a working solution, 0.1 ml of 1.0 M HCL was added to each 10 ml of working solution and air was bubbled vigorously into the solution through a piece of fine plastic tubing attached to an aquarium aeration pump. Bubbling lasted 20 minutes. The working

solution was then neutralised with 0.1 ml of 1.0 M NaOH per 10 ml of solution, sterilised by filtration through a 22 μm membrane filter in an in-line holder attached to a syringe, and finally dispensed aseptically, in small volumes, into sterile glass, screw capped vials. Each vial contained sufficient solution for one range of concentrations in a heterotrophic uptake experiment.

For all the work on Lake Rotomanuka it was considered sufficient to store the vials of substrate frozen without further sterilisation and to rethaw each immediately prior to use. Substrate kept in this manner presented no problems with respect to high $^{14}CO_2$ levels. Because of uncertainties regarding transport and storage in Antarctica, substrate for use in the work on Lake Fryxell was further sterilised by autoclaving. In Antarctica it was stored predominantly at a temperature of 0-5°C. The $^{14}CO_2$ content of the Na-[1-^{14}C]-glycolate increased slowly with time but was never greater than 10% of any experimental value for $^{14}CO_2$ release. The $^{14}CO_2$ of the D-[U-^{14}C]-glucose was higher, sometimes accounting for 30% of experimental values, but comparison of the $^{14}CO_2$ content of substrate which had been frozen immediately after autoclaving and had remained frozen in New Zealand throughout the period of the Antarctica field work, with the $^{14}CO_2$ content of substrate returned from Antarctica and with the $^{14}CO_2$ expelled from substrate in Antarctica showed that these high blank values remained constant and were probably due to autoclaving. ^{14}C-glucose which was not autoclaved did not contain high $^{14}CO_2$ levels. No explanation is advanced for the loss of CO_2 from glucose upon autoclaving. Loss of the carboxyl group of glycolate could explain the phenomenon for glycolate. Whatever the explanation, it seems that filter sterilisation followed by freezing is preferable to autoclaving as a means of maintaining substrate sterility.

Deciding on the actual range of substrate concentrations to be used in a heterotrophic uptake experiment involves a compromise between three important factors.

(i) The concentration range must be kept as low as possible, preferably within the same order of magnitude as natural substrate concentration. This is to ensure that the kinetics measured are those of the organisms which are responsible for

uptake of the naturally occurring substrate (see section 1.2.4 (d)). Another important consideration in this respect is that if all data points in an uptake experiment are close to saturation (V_{max}) any experimental error is likely to have a large effect on extrapolation to produce T_t and $(K_T + S_n)$, Krambeck (1979) (see figure 1.2/1).

(ii) Sufficient substrate must be present so that countable levels of ^{14}C-substrate are incorporated into biomass or converted to $^{14}CO_2$ at the lowest substrate concentration, within the incubation time used.

(iii) The incubation time must be short enough so that the heterotrophic organisms being studied do not have a chance to respond to the increased substrate presented by the experimental conditions by increasing their uptake capabilities by induction of transport systems or by cell division. It must also be short enough so that substrate depletion is insignificant. Substrate depletion would result in a severe departure from the assumptions used in the derivation of the Michaelis-Menten equation. Thompson and Hamilton (1974) give a figure of 5% as being a generally accepted, maximum allowable substrate depletion. In this work I found that substrate depletion was not a problem.

Ultimately, choice of substrate concentrations was based on a compromise between a concentration of substrate of the same order as the natural concentration and that giving significant uptake within an acceptable incubation time.

In all experiments the working solutions were at a ^{14}C concentration of approximately 40,000 Bq ml^{-1} (37,000 Becquerel (Bq) = 1 microCurie (μCi)) from which a range of volumes from a minimum of 25 μl to a maximum of 400 μl were added to 10 ml aliquots of lake water, giving a maximum 4% dilution of the lake water. The resulting minimum added substrate concentrations were 0.6 μg carbon l^{-1} for glucose, which had a specific activity of 1.21×10^{10} Bq $mmole^{-1}$ (327 mCi $mmole^{-1}$) and 13 μgCl^{-1} for glycolate which was available only at the relatively low specific activity of 1.85×10^{8} Bq $mmole^{-1}$ (5 mCi $mmole^{-1}$). Some literature values for

natural concentrations of glucose are 0.7 to 70 μgCl^{-1} (Vaccaro, Jannasch, Hicks and Carey, 1968) and 2 to 20 μgCl^{-1} (Wood and Chua, 1973) and values for glycolate are generally in the order of a few tens of μgCl^{-1} (various sources, see section 3.2.4.5).

2.2.2 Substrate Addition and Incubation Duration and Conditions

For each location and time of year an appropriate incubation time was calculated from the results of the measurement of uptake in water samples at a single concentration of each substrate, over a range of times. A time was chosen which would give a total uptake of approximately 30 Bq (after blank subtraction) at the lowest substrate concentration. If the lowest concentration was not used for determining the relationship between uptake and time it was assumed that uptake varied linearly with concentration, for the purposes of calculating a suitable incubation time. With a ^{14}C counting efficiency of approximately 50%, 30 Bq gave 15 counts per second (cps), taken up, which was subdivided into a respired fraction and an incorporated fraction. In Rotomanuka approximately 80% of glycolate which had been taken up in experiments was respired. Thus the smaller fraction of ^{14}C activity was associated with the incorporated ^{14}C on the filter and would be approximately 20% of 15 cps, or 3 cps. The background of the liquid scintillation counter was 0.7 ± 0.3 cps (30 times; 1,000 counts each) which gave an upper 95% confidence limit of 1.3 cps (2-tailed students-t-distribution). Thus, incubation times were chosen to produce barely significant counts in the least active sample. This was in the interests of maintaining incubation times short in comparison with the 24 hour period being examined. In fact, because the relationship between concentration and uptake was never linear, incubation times were long enough to provide uptake values, in the lower concentrations, which were higher above background, than calculated.

In Lake Rotomanuka incubation times were in the range of one to three hours. When large numbers of subsamples were incubated simultaneously, as on 3-4 April 1981, it was necessary to use a range of incubation times for experimental convenience. In Lake Fryxell a standard incubation time of six hours was necessary. The effects of this are discussed in section 4.4.1.

Measurement of the effect of time on uptake served a second purpose. By choosing an incubation time over which there was a linear relation-

ship between uptake and time it was possible to ensure that the organisms involved were not modifying their uptake capability within the incubation time, or times, chosen. For example, an increase in population size or an increase in V_{max} per cell as a response to the added substrate would have been seen as an increase in the slope of the graph of uptake against time. Unfortunately, it was not possible to measure uptake over the first few minutes of incubation. This would have required substrates of extremely high specific activity, or alternatively, unmanageably large samples. One possible effect of the experimental conditions used which can not be ruled out and which might have severe consequences on the interpretation of the results of this investigation is the induction of increased uptake ability within the first few minutes of incubation.

Apart from added substrate, other important factors influencing experimental conditions were (a) light, (b) temperature and (c) agitation.

(a) Light

All incubations were carried out in the dark. The waterbaths used to maintain incubation temperature were kept in a cardboard box inside a shed, in the field. Because ambient light levels beneath the ice in Lake Fryxell were only 0.25% of surface irradiance, at the sampled depths (Harfoot, *pers. comm.*) darkness was further ensured for these samples by wrapping each individual incubation syringe in tin foil.

Darkness was necessary to ensure that no photoassimilation of substrate by algae could occur during daylight hours. Photoassimilation is a process by which some algae can utilise extracellular carbon compounds for growth but only in the presence of light (see the review of Droop, 1974). Although it is generally assumed that algal uptake of organic compounds is negligible at the concentrations of those compounds found in natural waters, it was felt that uniform lighting conditions would remove the risk that observed changes in uptake kinetics could be due to changes in the availability of light rather than substrate. The effect of light on uptake was only tested in Lake Fryxell where uptake was significantly higher in the light than in the dark (see section 4.4.2.4).

(b) Temperature

Some literature values for the Q_{10} for substrate uptake by aquatic heterotrophic populations are 3.5 for glucose (Hodson and Azam, 1979), 2.2-4.1 for glucose and 2.8 for acetate (Wright and Hobbie, 1966) and between 2 and 3 for acetate (calculated from data in Hobbie and Rublee, 1977). Witzel (1980) determined glucose uptake kinetics in natural water samples at different temperatures in order to find temperatures which were optimal for uptake. His results show that V_{max} can decrease to 50% of its maximum at only 5°C above its optimum temperature.

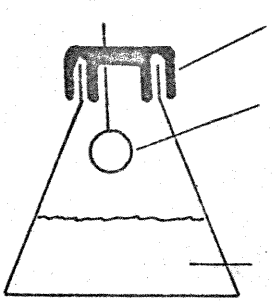
Fortunately, in the lakes studied, diurnal temperature changes were slight, with no change in Lake Fryxell and a maximum of 1°C at 1m depth in Lake Rotomanuka. Incubations were carried out within a few degrees of lake temperature and throughout each incubation, temperature was kept constant within ± 1°C by the use of a well-insulated polystyrene waterbath. The same waterbath temperature (± 1.5°C) was used throughout any given 24 hour period.

(c) Agitation

Theoretically agitation may increase measured V_{max} if diffusion of substrate to a cell is more limiting to uptake than the uptake process itself. This is covered, theoretically, by Koch (1971) and Pasciak and Garvis (1974). No attempt was made to determine the effect of agitation on uptake. In the interests of uniformity, all incubations were carried out without agitation.

2.2.3 CO_2 Displacement and Trapping

Parsons and Strickland (1961) used only filterable ^{14}C-activity as a measure of uptake of ^{14}C-labelled compounds. Since the development of a simple technique for trapping and counting respired $^{14}CO_2$, by Hobbie and Crawford (1969), it has become customary to measure total uptake as the sum of respiration and incorporation into biomass. However, this is not always so. Witzel (1980), whilst studying the effect of temperature on uptake kinetics, measured only incorporation of ^{14}C-label into biomass. For any investigation into changes in



septum seal

25mm diameter disc of glass fibre paper saturated with 0.2ml of 50:50 v/v 2-phenylethylamine/methanol. Disc removed to stop experiment

25ml flask containg 10ml water plus Na_2CO_3 + $NaH^{14}CO_3$. Acidified to start experiment by injecting 0.2ml H_2SO_4 through septum seal

The graph shows the effect that the time between the addition of acid and the removal of the filter paper disc had on the recovery of added $^{14}CO_2$

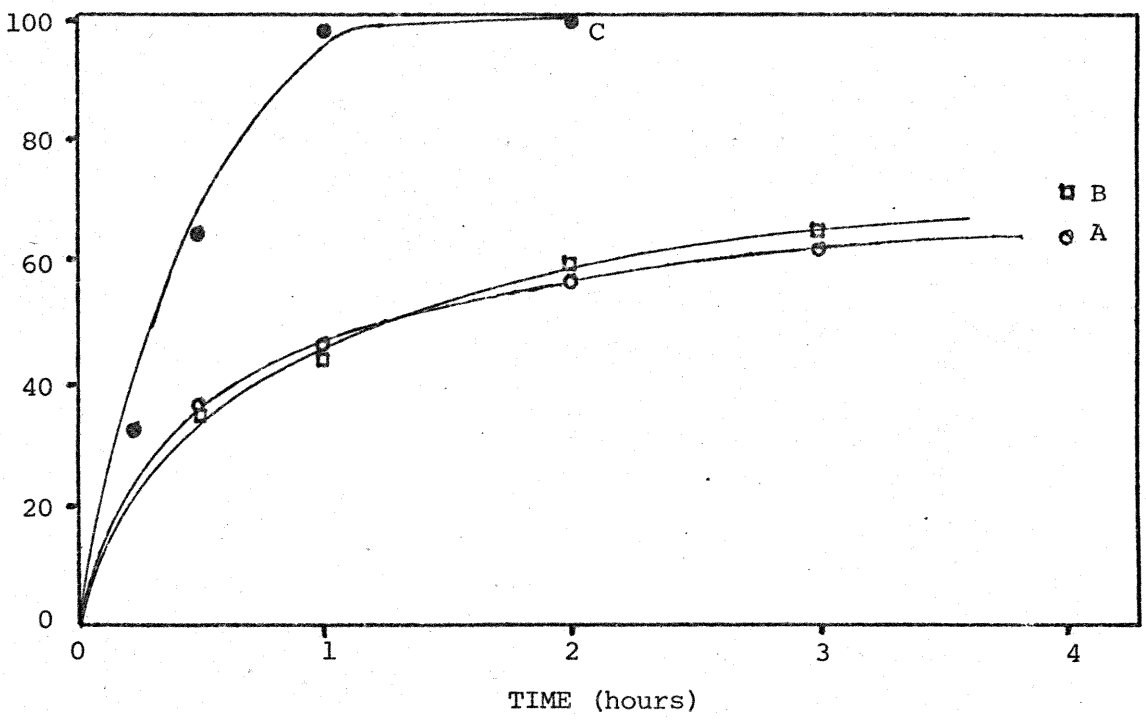


Figure 2.2/2 - The Effect of Time on the Recovery of $^{14}CO_2$ from Solution in a Closed Flask

Refer to diagram above and section 2.2.3 for explanation

A - $^{14}CO_2$ in tapwater; B - $^{14}CO_2$ in 500 mgl^{-1} Na_2CO_3;

C - $^{14}CO_2$ in 100 mgl^{-1} Na_2CO_3 with agitation

kinetics, as opposed to investigations using kinetics to quantify carbon flow, it could be argued that useful results can be obtained by measuring the kinetics of either anabolism, or catabolism, rather than both. However, it was felt that for the purposes of this investigation, it would be more useful to measure both as a change in the division of substrate between anabolic and catabolic pathways might be an important response to changing substrate availability.

The method of Hobbie and Crawford is essentially as follows. Samples are incubated in a flask closed with a septum cap from which hangs a piece of filter paper. Incubation is terminated by injecting acid into the sample through the septum cap. The acid displaces CO_2 from solution and this is trapped by a trapping agent such as KOH solution or 2-phenylethylamine which is injected through the septum cap to saturate the filter paper. The major disadvantage of this technique, as far as my work was concerned is that, unless the flasks are shaken CO_2 displacement is extremely slow (see figure 2.2/2). As it was intended to perform incubations in the field where a shaker would not be available, particularly in Antarctica, it was decided that a faster and more portable technique for displacing and trapping CO_2 was needed. To return fixed samples to the laboratory for CO_2 displacement would have involved two major problems; (a) long and variable periods of time in which samples are in contact with fixative. Some fixatives, particularly acids, cause loss of labelled metabolites from cells. (See Ramsay (1976) and the review of Wright (1971)). (b) A wick would have to be inserted after transport resulting in possible loss of $^{14}CO_2$. If the wick was present during transport the risk of contamination with ^{14}C-substrate from the sample would be great.

The following is a discussion of the development of the technique which was ultimately used for recovering $^{14}CO_2$ from samples. It is based on a technique described by Thompson and Hamilton (1974) in which air is first bubbled through the acidified sample, then passed through a tube to a scintillation vial where it is bubbled through a CO_2 trapping mixture after which it is returned via another tube to the sample container and bubbled through the sample again. This closed loop system proved to be extremely fast, resulting in 97% recovery of added CO_2 in five minutes. However, each closed loop required a separate pump for air circulation and the technique as a whole required a power supply.

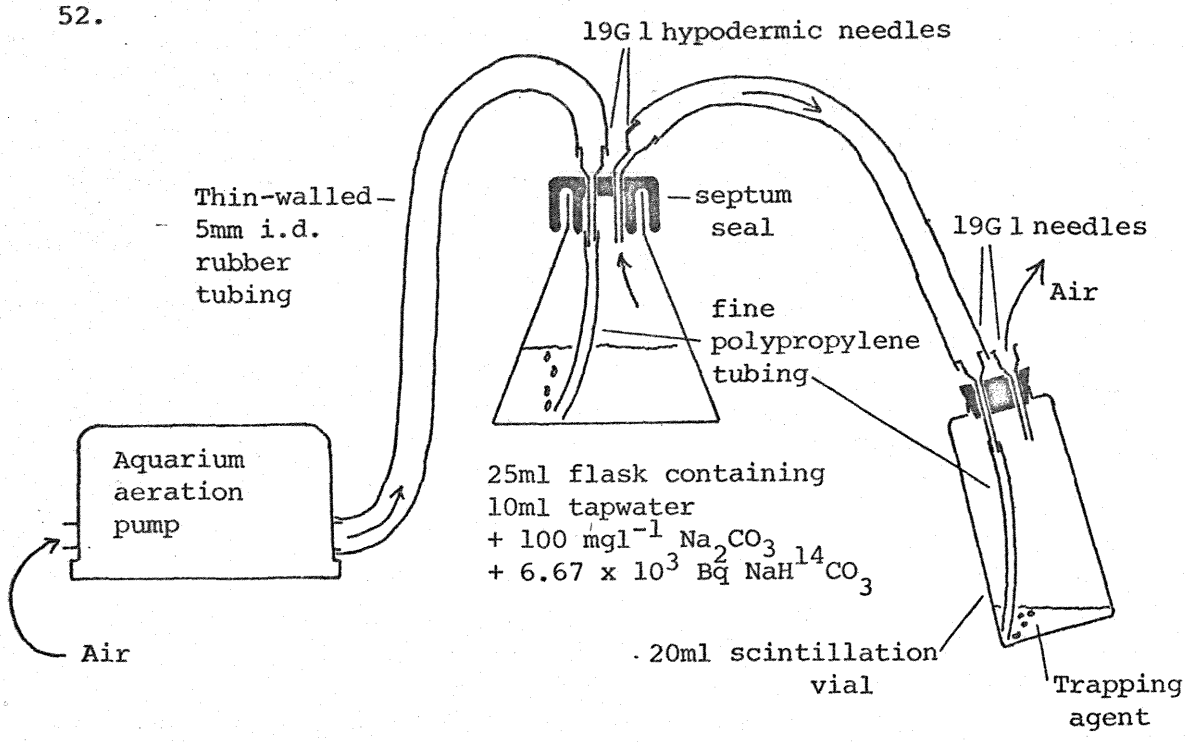


Figure 2.2/3 - Apparatus used to Test the Effectiveness of the "Open-Loop Technique" for $^{14}CO_2$ Recovery

The experiment was started by injecting 0.2ml of 2M H_2SO_4 through the septum seal into the flask and turning on the pump. At measured intervals the scintillation vial was changed.

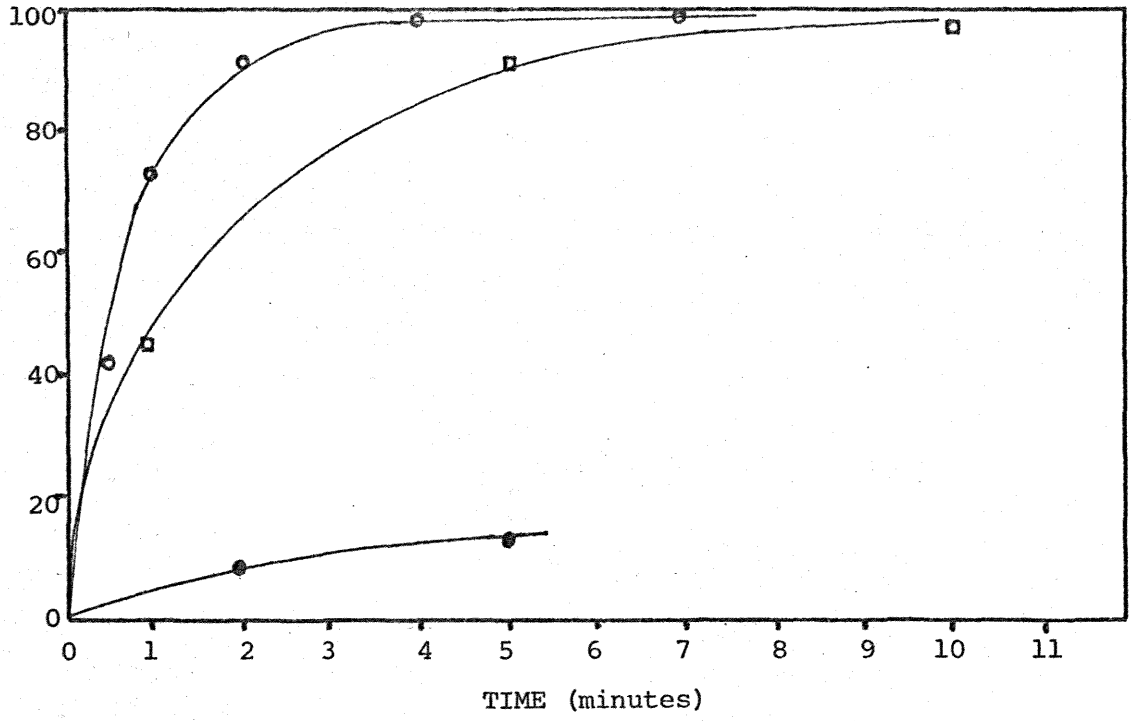


Figure 2.2/4 - The Effect of Sparging Time on the Recovery of $^{14}CO_2$ from Solution (100 mgl^{-1} Na_2CO_3) Using the "Open-Loop Technique"

See text (section 2.2.3) and figure 2.2/3 for explanation

A - in 0.4ml of 50:50 v/v β-phenylethylamine:methanol
B - in o.2ml of 50:50 v/v β-phenylethylamine:methanol
C - in 0.4ml of β-phenylethylamine in 10ml of scintillation cocktail

A preliminary experiment was conducted to test the necessity for a closed loop system as opposed to a simple open loop design which could theoretically allow an unlimited number of samples to be treated at the same time with a single air supply. The experimental system illustrated in figure 2.2/3 was used. The experiment was started by injecting 0.2ml 1.0M H_2SO_4 into the flask then turning on the pump. At intervals the pump was switched off and the scintillation vial changed. At the end of the experiment scintillation cocktail was added to each vial and recovered $^{14}CO_2$ activity was determined by liquid scintillation counting. The results for three different trapping mixtures are expressed in figure 2.2/4. Quite clearly the open loop system worked very well.

The Open Loop Technique Refined

After considerable experimentation with different arrangements of various types of incubation and trapping vessels, the arrangement shown in figures 2.2/1 and 2.2/5 was chosen. During incubation the syringe was sealed using a needle blocked with a silicone rubber plug. At the end of incubation this needle was removed and immediately replaced with a tube containing glass fibre filter paper soaked in the CO_2 trapping mixture. 0.2ml of 0.1M H_2SO_4 was injected through the septum seal, the valve connecting the air reservoir (car innertube) to the hypodermic needles was opened and a hypodermic needle was inserted through each suba seal into each syringe. A combination of the height of the water column in the pressure regulator and the bore and length of the needle maintained a uniform, predetermined air flow rate. After 15 minutes the hypodermic needles were removed and the air supply shut off. The saturated filter paper was blown into a scintillation vial using a burst of compressed air from a 20ml syringe. This avoided handling the filter paper with the possibility of cross contamination between filter papers from different samples. Retention of $^{14}CO_2$ on the glass tube was less than 1% of $^{14}CO_2$ trapped on the filter paper. Between samples, each hypodermic needle was blown through with compressed air from a syringe to remove moisture which might build up and impede flow.

Plate 2.2A shows a number of samples being treated simultaneously. Any number up to 18 could be treated at the same time on the stand shown simply by adding extra outlets, each one consisting of a plastic t-connector, a short length of rubber tube, the male fitting from the end of a 1.0ml syringe and a 23G 5/8 hypodermic needle.

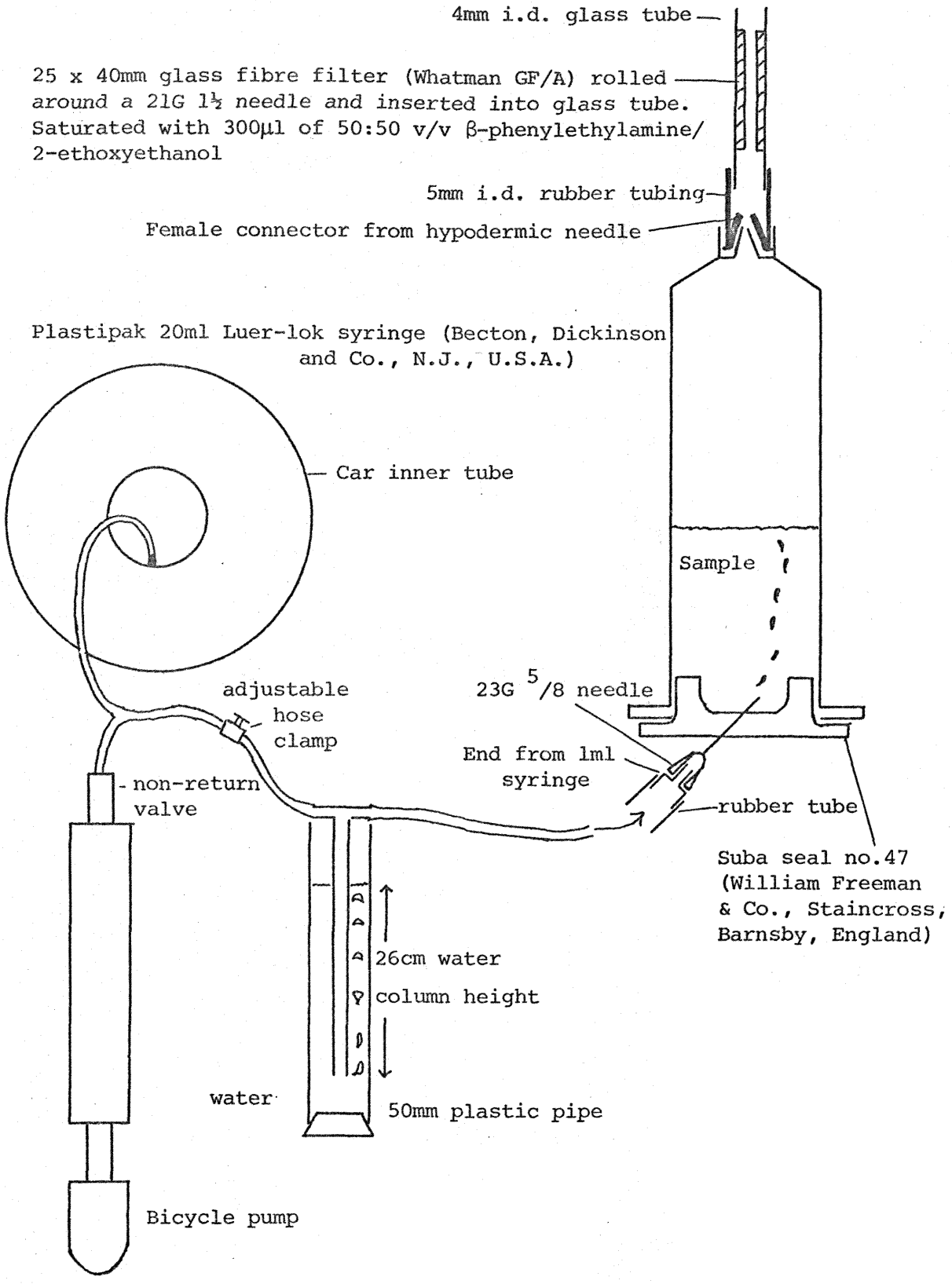


Figure 2.2/5 - Apparatus used in Refined "Open-Loop Technique"

(see section 2.2.3)

Not to scale

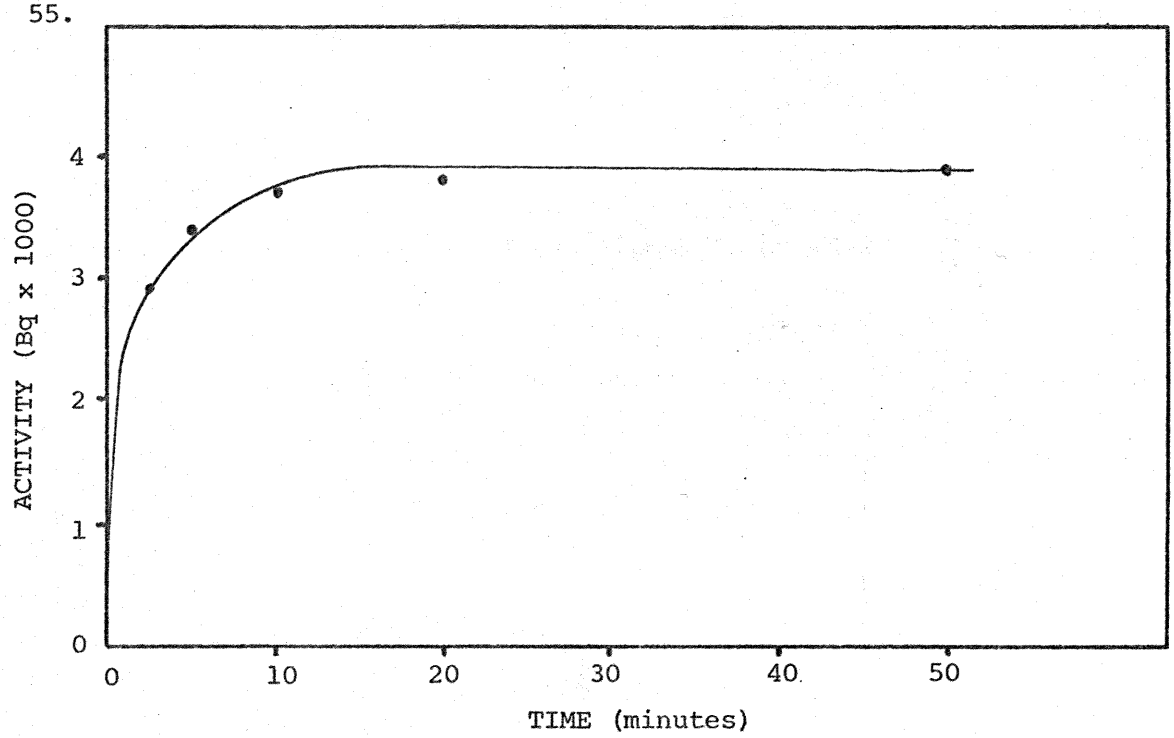


Figure 2.2/6 - The Effect of Time before Addition of Scintillation Cocktail on the Stabilisation of the ^{14}C-activity of 50µl of $NaH^{14}CO_3$ (approximately 80,000 Bq ml^{-1}) to 300µl of 50:50, v/v, β-phenylethylamine/2-ethoxyethanol

(see section 2.2.3(a))

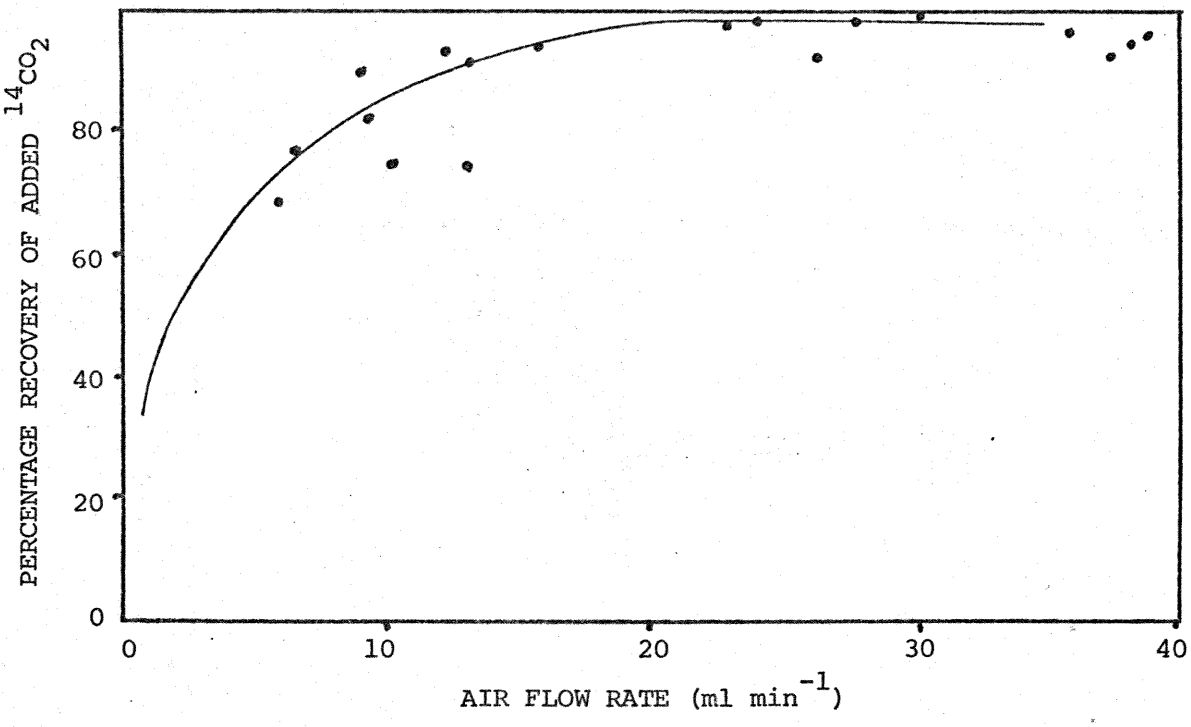


Figure 2.2/8 - The Effect of Air Flow Rate on the Recovery of $^{14}CO_2$ using the Equipment Illustrated in figure 2.2/5 and Sparging with Air for 10 minutes

(see section 2.2.3(d) for an explanation)

In the development of this technique the following aspects were considered.

(a) Measurement of the ^{14}C activity of $NaH^{14}CO_3$

Two difficulties were found to be associated with the calibration of the efficiency of $^{14}CO_2$ recovery using $NaH^{14}CO_3$ (Radiochemical Centre, Amersham) as a standard: (i) that of decreasing activity in the standard with time due to CO_2 loss to the atmosphere and (ii) that of the insolubility of CO_2 in some scintillation solvents.

The problem of CO_2 loss was overcome by the addition of 1-2 drops of 5M NaOH to 10ml stocks of dilute $NaH^{14}CO_3$ (80,000 Bq ml^{-1}).

The problem of insolubility of CO_2 in the scintillation cocktail was resolved by following the suggestion of Iverson, Bittaker and Myers (1976) who mixed $NaH^{14}CO_3$ with an excess of 2-phenylethylamine to form a carbamate which was stable in scintillation cocktail. I used 50 µl of an aqueous solution of $NaH^{14}CO_3$ in 150 µl of 2-phenylethylamine which was diluted with a further 150 µl of either methanol or 2-ethoxy-ethanol, to reduce the viscosity of the 2-phenylethylamine, to simplify handling (Woeller, 1961). ^{14}C-activity increased with the time which passed before cocktail was added (see figure 2.2/6). Maximum activity was not reached but in all cases $NaH^{14}CO_3$ standards were left to stand in the 2-phenylethylamine mixture for at least 60 minutes before addition of cocktail. Estimates of $^{14}CO_2$ recovery efficiencies are probably exaggerated by several per cent because of incomplete measurement of added $NaH^{14}CO_3$. The ^{14}C-activity of $NaH^{14}CO_3$ standards counted in this way was stable for at least several days with or without cocktail.

(b) Acidification

In all samples, from Lake Rotomanuka, 0.2ml of 1.0M H_2SO_4 was used to acidify each 10ml sample, to facilitate the purging of CO_2. This was the quantity and type of acid used by Hobbie and Crawford (1969a & b), and was sufficient to lower pH to approximately 2. For all samples in which total CO_2 levels

were high, including samples from Lake Fryxell and samples with added $NaCO_3$ used to test the effect of CO_2 concentration on $^{14}CO_2$ trapping efficiency, sufficient H_2SO_4 was added to lower the sample pH to 2. pH was measured with a pH test paper (BDH pH range 1-4).

No attempt was made to assess the extent of loss of ^{14}C-compounds from cells due to acidification, nor to reduce this loss by cooling in an ice bath (Ramsay, 1976).

(c) Choice and Preparation of Trapping Agent

Since its introduction as a CO_2 trapping agent by Woeller (1961), 2-phenylethylamine has achieved almost universal acceptance as the trapping agent in heterotrophic uptake work. It is superior in trapping efficiency to more traditional trapping agents such as Hyamine hydroxide, KOH and NaOH (Woeller, 1961). In liquid scintillation counting it causes less quenching than Hyamine hydroxide, if redistilled before use, and does not create chemiluminescence problems as NaOH does (Iverson, Bittaker and Myers, 1976).

Because of the relative cheapness of KOH and its ease of preparation, its suitability as a trapping agent was tested. The first test involved its compatibility with the toluene/ethanol/2-ethoxyethanol scintillation cocktail used for counting. When 200 µl of 20% KOH was added to 10ml of cocktail containing D-$\left[U\text{-}^{14}C\right]$-glucose, an immediate loss of 5% of ^{14}C counts was noticed. ^{14}C counting efficiency decreased to 86% of its unquenched value in eight hours then to 74% in 96 hours. Although the initial loss in activity is not severe, none of the loss in activity could be accounted for by the sample channels ratio quench correction technique in use (see section 2.2.6). If, as happened occasionally during the course of my work, 100 samples are counted for 10 minutes each, the final sample is not counted until 17 hours after the first. Using KOH as a trapping agent could result in a 20% variation in counting efficiency between the first and last sample, which could not easily be corrected for. KOH was discounted as a trapping agent, without any further investigation into its effect on ^{14}C counting. It is possible that the

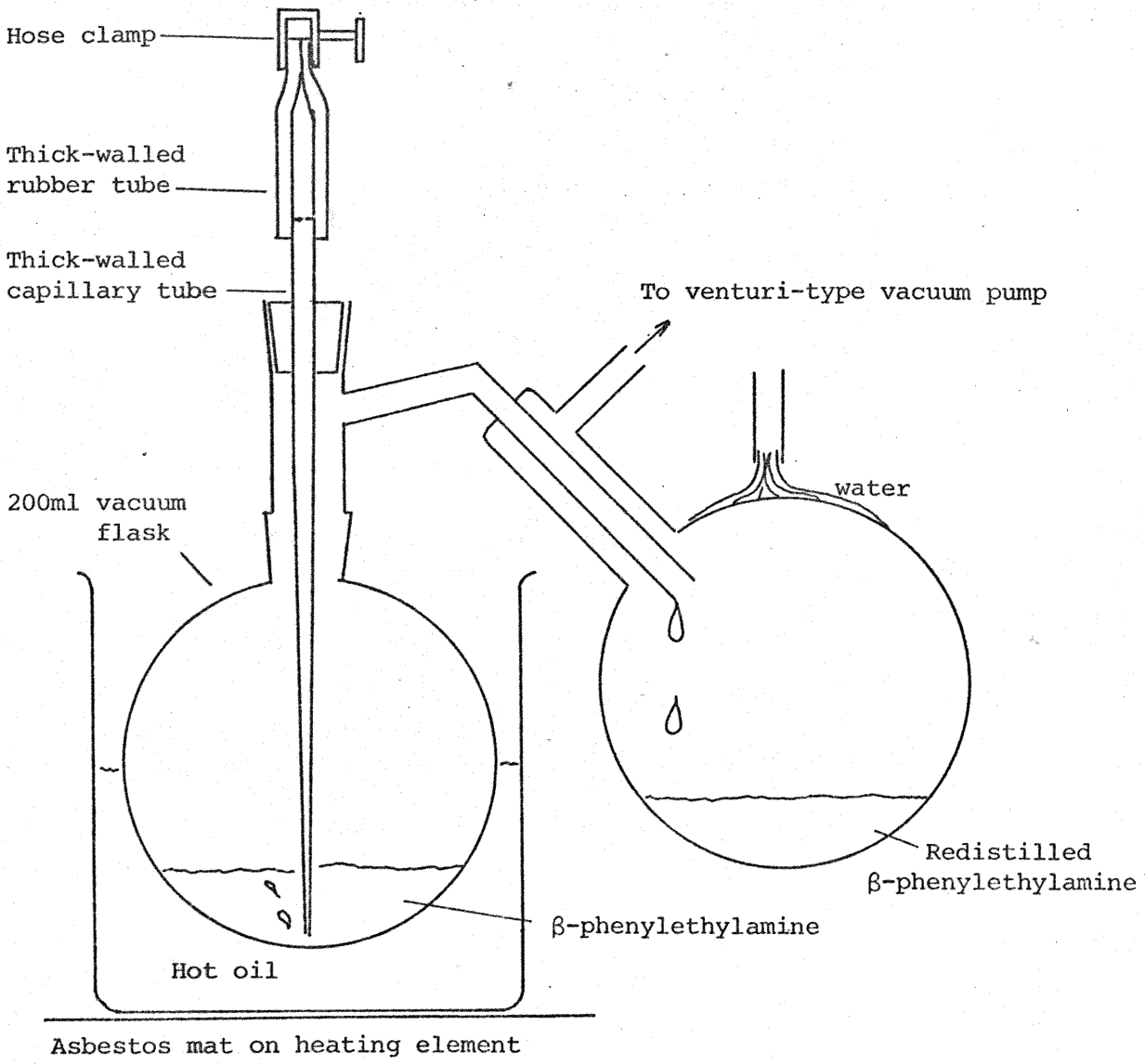


Figure 2.2/7 - Redistillation of β-phenylethylamine

(see section 2.2.3(c))

All glassware is Quikfit except thick-walled capillary tube which was custom made by the University Glassblower

lost counts were a result of initial chemiluminescence. 200 µl of redistilled 2-phenylethylamine also caused a 5% drop in counts per second in 10ml of scintillation cocktail containing D-[U-^{14}C]-glucose. However, this effect was attributable to normal quenching and could be corrected using the sample channels ratio method. Moreover, initial loss of counting efficiency was not followed by any subsequent loss.

2-phenylethylamine (pure, Koch Light Laboratories, Colnbrook, England) was used in all subsequent work. Redistillation of new stocks was recommended by Kobayashi and Maudsley (1974) on the grounds that this renders the carbamate more soluble in toluene based cocktails. It is also necessary to remove the yellow quenching substance found in aged 2-phenylethylamine (Iverson, Bittaker and Myers, 1976). Vacuum distillation was used as recommended by Oliverio *et al* (1962). A simple distillation set up using Quikfit glassware was used. The air bleed capillary used to prevent 'bumping' was custom made by the University glassblower. The water venturi pump used would not create a low enough vacuum to enable distillation in a boiling water bath; consequently an oil bath was needed. See figure 2.2/7.

For CO_2 trapping, redistilled 2-phenylethylamine was diluted with an equal volume of either solvent grade methanol or scintillation grade 2-ethoxyethanol. Woeller (1961) used methanol because it lowers the vapour pressure of the 2-phenylethylamine and reduces evaporation and because it reduces viscosity and allows easier handling. 2-ethoxy-ethanol was found to be an excellent solvent for the carbamate produced by the reaction of CO_2 with 2-phenylethylamine (Brown, 1979). Because insoluble carbamates were found to lead to blocking of the CO_2 trapping 'chimney' shown in figure 2.2/5, a 2-phenylethylamine : 2-ethoxyethanol mixture was used instead of a 2-phenylethylamine : methanol mixture.

(d) Choice of Air Supply and Flushing Time

By altering water column height and/or needle gauge and length in the basic set up illustrated in figure 2.2/5, it was possible to control the air flow through a sample. Air flow was measured with a simple bubble gauge and stopwatch. The effect of air

flow rate on the flushing of $NaH^{14}CO_3$ from tap water is shown in figure 2.2/8. In each case air flow lasted for 10 minutes. Except for needle size and water column height all other conditions were as shown in figure 2.2/5. A column height of 26cm coupled with a 25G 5/8 needle, giving a flow rate of 36.0 ± 2.9 ml min^{-1} (mean ± standard deviation) was chosen. This flow rate gave a reasonable margin for error. The 25G 5/8 needle was fine enough to allow repeated piercing of a septum seal without permanent damage but was not so fine that bending and complete blockage were a problem as they were with a 30G ½ needle.

The car innertube air reservoir was sufficiently large to displace CO_2 from 10 samples before further pumping was required. This left the operator's hands free during the flushing period, a considerable advantage under field conditions. The components of the air supply, i.e. the bicycle pump, valve, innertube, and water column (see figure 2.2/5) were all robust and relatively fool proof and caused no problems in the field.

In practice, a flushing time of 15 minutes was used rather than the 10 minutes used in calibrating the system. This allowed an extra margin for error. The main problem experienced with the CO_2 recovery technique described was with decreased flow rate due to either blockage of needles with water, if the air supply was shut off before the removal of the needles in previous usage, or to blockage with rubber from the septum seal. In each case, blowing the needles through with compressed air, from a syringe, between samples, solved this problem.

To test the effect of running more than one sample from the same air supply, five 10ml samples, consisting of tap water plus $NaH^{14}CO_3$ standard, were flushed in parallel. This was done twice, the first set giving a mean of 93.2% recovery with a standard deviation of 1.9% and the second set giving a mean of 94.2% recovery with a standard deviation of 1.9%.

(e) Choice of Filter Paper Support for Trapping Agent

The use of cellulose fibre filters as wicks in the CO_2 trapping

technique of Hobbie and Crawford (1969) has resulted in varying degrees of quenching during scintillation counting. Up to 75% quenching was reported by Booth (1978). Apelgot and Duquesne (1976) suggest that cellulose fibres used as supports for labelled compounds actually absorb some of the label so that it is not in contact with the scintillation cocktail. This, coupled with the opacity of cellulose fibres results in low counting efficiency. Glass fibres filters are recommended by Apelgot and Duquesne, as the fibres are non-absorbent and almost transparent to the emitted light.

(f) Effect of CO_2 Concentration on Recovery

Wetzel (1975) states that the ΣCO_2 concentration of freshwaters around the world are typically within the range of .05 - 10 mmole l^{-1}, or 2.2 - 440 mg l^{-1} ($\Sigma CO_2 = CO_2 + HCO_3^- + CO_3^{2-}$). Iverson, Bittaker and Myers give the CO_2 adsorption capacity of 2-phenylethylamine as 0.2g ml^{-1}. Thus, 150 µl of 2-phenylethylamine has a capacity of 30mg CO_2 which, if dissolved in 10ml of lake water would give a ΣCO_2 concentration of 3,000 mg l^{-1}. For the maximum ΣCO_2 concentration likely to be found, it can be seen that the amount of 2-phenylethylamine used is roughly six times in excess of what should be required. However, the effect of ΣCO_2 concentration on the recovery of $^{14}CO_2$ was tested.

Na_2CO_3 was added to Milli-Q reagent grade water (Millipore Corporation, Bedford, Mass. U.S.A.) to give a range of ΣCO_2 concentrations. The recovery of $NaH^{14}CO_3$ standards was measured using sufficient acidification to lower the pH to 2 and a flushing time of 15 minutes. The results are expressed in figure 2.2/10. Obviously the CO_2 capacity of the 2-phenylethylamine is not the sole limitation to CO_2 recovery. The $NaH^{14}CO_3$ added was at a specific activity of >1.5 GBq/mmol (manufacturer's data) or $<3 \times 10^{-4}$ mg CO_2 per 10,000 Bq. Thus, the 10,000 Bq used as a tracer would mot raise the concentration by more than .03mg l^{-1}. Displacement of the CO_2 by acidification and bubbling was, likewise, not the problem. Using the same displacement technique but trapping CO_2 by bubbling it through fine plastic tubing into a mixture of 0.5ml of 2-phenylethylamine and 1.0ml 2-ethoxy-ethanol, in

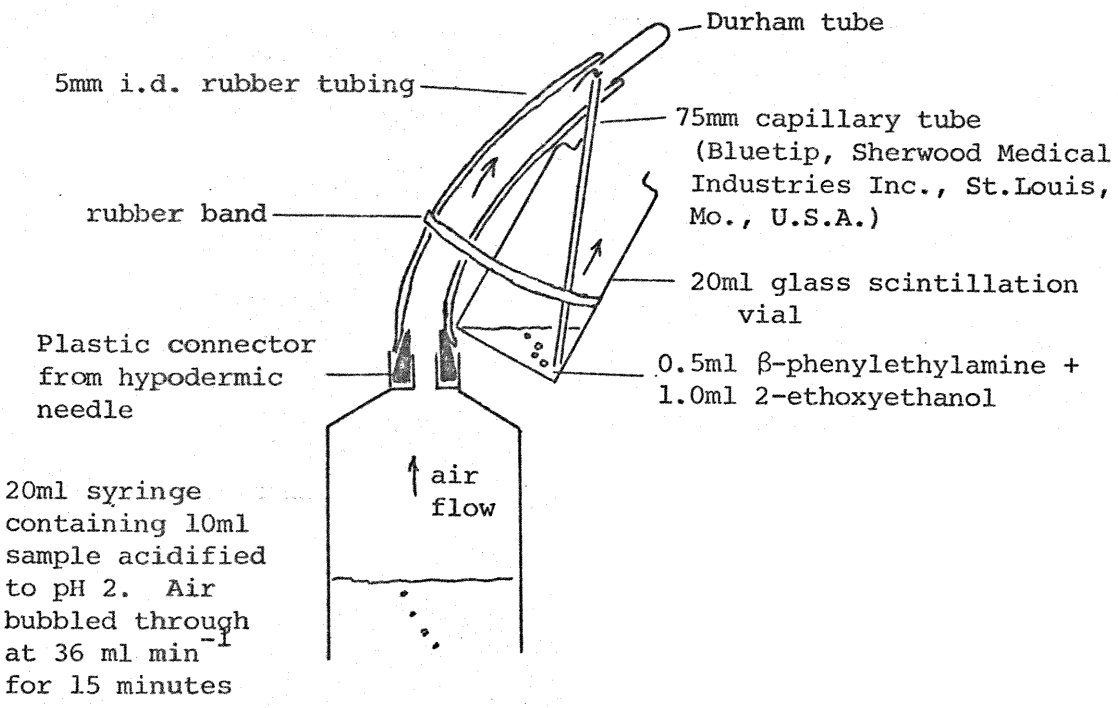


Figure 2.2/9 - Modification of the Apparatus used to Recover $^{14}CO_2$ after Flushing from Samples

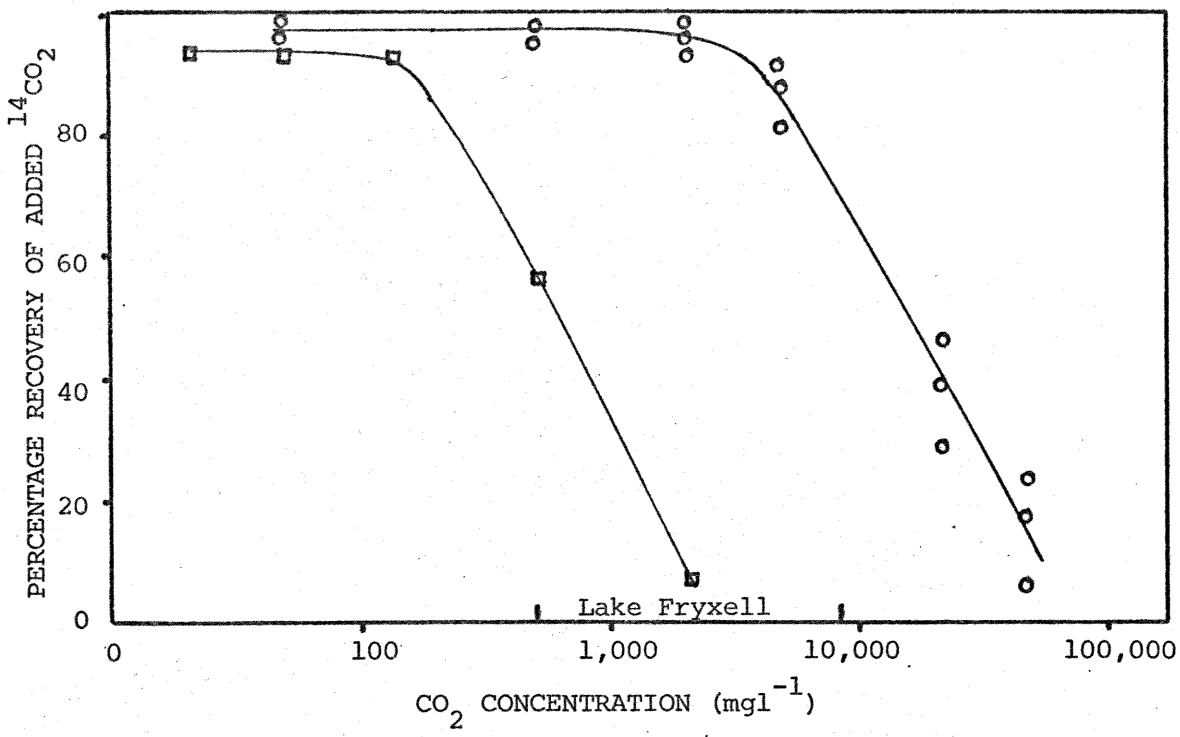


Figure 2.2/10 - Recovery of $^{14}CO_2$ from Solutions Containing Different Concentrations of CO_2 (as Na_2CO_3)

A - Using the technique shown in figure 2.2/5

B - Using the modification shown in figure 2.2/9

In each case 10ml of solution containing $NaH^{14}CO_3$ and Na_2CO_3 was acidified to pH 2 and sparged with air for 15 minutes. (See section 2.2.3(f) for further details)

a scintillation vial, resulted in 95% recovery of added $^{14}CO_2$ at a ΣCO_2 concentration, in the 10ml sample, of 2,200 mg l^{-1}. From this it was assumed that the major limitation, to CO_2 recovery, of the 2-phenylethylamine : 2-ethoxyethanol soaked filter paper 'chimney', was the limited surface area exposed to passing CO_2 and the slow replacement of 2-phenylethylamine at this surface, by diffusion. The reduced CO_2-trapping capacity of 2-phenylethylamine used in this way was not a limitation in Lake Rotomanuka. There, $^{14}CO_2$ recovery was 92% ± 7% (mean ± standard deviation, n=3) on 13/9/80 and 94% ± 3% (n=5) on 2/3/81. However, Lake Fryxell is reported to have a ΣCO_2 content which is higher than the maximum of the range given by Wetzel. In the summer of 1979-1980 the CO_2 concentration ranged from 440mg l^{-1} to 8,800mg l^{-1}, with concentrations increasing with depth and increasing as summer progressed. At 9m the ΣCO_2 concentration went from 1,200mg l^{-1} in early summer to 1,700mg l^{-1} in late summer. ΣCO_2 levels were measured by displacement with acid and analysis with an infra-red gas analyser (M. Lawrence, *pers. comm.*).

A new technique was devised for trapping displaced CO_2. See figure 2.2/9 for details. In this technique bubbling rapidly mixes the trapping mixture so that a greater proportion of 2-phenylethylamine is available for CO_2 trapping than in the chimney technique. The effect of ΣCO_2 concentrations up to 44,000mgl^{-1} (1.0M Na_2CO_3) is given in figure 2.2/10. After CO_2 displacement and trapping the capillary tubes are broken and dropped into their respective scintillation vials. A new capillary is used for each sample to avoid carryover of activity between samples. In Lake Fryxell water, from 9m depth, this technique gave a $^{14}CO_2$ recovery efficiency of 95% ± 4% (mean ± standard deviation, n=4).

(g) Stability of $^{14}CO_2$ Activity in Trapping Agent and Cocktail

Thompson and Hamilton (1974) mention the possibility of loss of activity, with time, from $^{14}CO_2$ samples trapped in 2-phenylethylamine. This, they said, could be prevented by using an excess of 2-phenylethylamine. During field trips, known amounts of $^{14}CO_2$, added as $NaH^{14}CO_3$, were flushed from lakewater in parallel with the heterotrophic uptake samples.

This was used to test efficiency of $^{14}CO_2$ recovery, but also tested the stability of CO_2 in the trapping mix. As efficiencies of $^{14}CO_2$ recovery in field samples were never significantly different from the values obtained in the laboratory, in which samples were counted within hours of the completion of recovery, it can be assumed that $^{14}CO_2$ was stable in the trapping mixtures used. In the case of the Lake Fryxell samples this stability lasted for 21 days.

The addition of cocktail to the trapping mixture resulted in some tendency for $^{14}CO_2$ activity to be lost. This could be demonstrated by the repeated counting of the same samples. In one set of ten samples which were recounted over a period of time, no sample lost more than 1% activity within 24 hours but the mean loss after five days was 4% with a range from 0.2% to 15%. Higher loss was, in all cases, associated with loose or faulty caps. Thereafter, faulty cap liners were discarded during washing and caps were tightened thoroughly after addition of cocktail. The problem of $^{14}CO_2$ loss was then ignored as the error it caused was insignificant in comparison with the scatter between results from identical natural samples.

(h) CO_2 Permeability of Plastic Syringes

The plastic syringes (20ml) used for incubation in the heterotrophic uptake technique were tested for CO_2 permeability by incubating syringes containing 10ml tap water and known amounts of $NaH^{14}CO_3$ in a waterbath at room temperature for up to 24 hours. The syringes were sealed with a septum cap and a silicone rubber blocked needle as they were during heterotrophic uptake experiments. No decline in $^{14}CO_2$ recovery was noticed up to 24 hours.

In summary, a technique was developed which enabled recovery, in the field, of $^{14}CO_2$ produced by respiration of ^{14}C substrates by aquatic bacteria. Recovery efficiencies were between 90 and 95% and with the apparatus described ten 10ml samples could easily be treated within half an hour. The equipment was robust, easily transported and required no power supply. A similar 'open loop' technique for recovering $^{14}CO_2$ from one litre lake water samples has since been described by Hesslein,

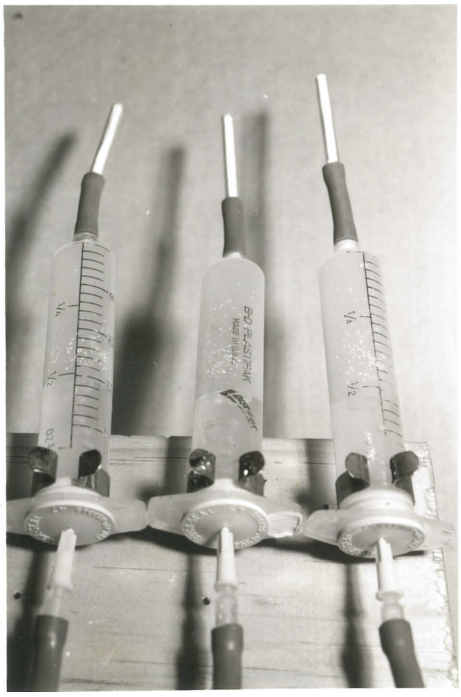
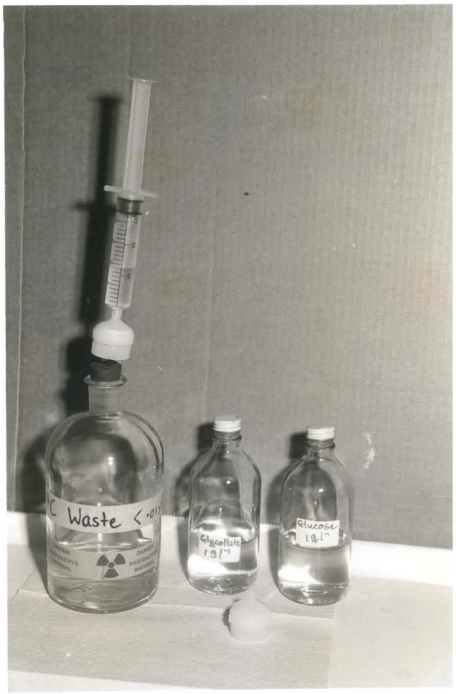


Plate 2.2A - The apparatus used for recovering $^{14}CO_2$ from samples, each showing 20ml syringe, inlet hypodermic piercing septum seal and 'chimney' containing trapping agent on rolled filter paper support (see section 2.2.3)

Plate 2.2B - Filtration of sample and washing of filter (see section 2.2.4)



Broecker and Quay (1979). However, this technique requires a helium gas supply and 70% recovery is only attained after 7-9 hours.

2.2.4 Filtration of Sample

After incubation and the recovery of CO_2, each sample was filtered as follows. A filter holder (Millipore Swinnex-25, Millipore Corporation, Bedford, Mass., U.S.A.) or a Sartorius Polycarbonate syringe filter holder, (Sartorius Gmbtl, Göttingen, W.Germany) holding a 25mm diameter, 0.2 µm pore size, cellulose nitrate membrane filter (Sartorius Catalogue No. SM11307) was attached to the Luer-Lok fitting at the end of the syringe. The septum seal was removed from the other end of the syringe and a plunger inserted. See plate 2.2B. Uniform positive pressure, of approximately 70 kPa, was applied by hand, by keeping a constant 10ml head space above the liquid. After sample filtration a further 10ml volume of an unlabelled solution of the substrate being investigated, was passed through the filter, again by using the syringe. This solution was at a substrate concentration of 1.0 gl^{-1} and was intended to remove, by isotopic exchange, any labelled substrate adsorbed to detritus, cell surfaces or the surface of the membrane filter. Filters were removed from the holders and stored, still damp, in sealed scintillation vials, for return to the laboratory for counting. The wash solution used contained 4% formaldehyde to preserve it and also to ensure that cells on the filter were not resuscitated by the washing off of the acid with which incubations were terminated.

Two errors associated with filtration of heterotrophic uptake samples have been reported in the literature. The first occurs when different volumes of sample are filtered. Arthur and Rigler (1967) noted in primary productivity experiments using $^{14}CO_2$ as a tracer, that the recovery of fixed carbon decreased on a unit volume basis with increasing volumes of sample filtered. This problem was also found to be associated with the use of ^{14}C organic tracers (Thompson and Hamilton, 1974). This problem is discussed by Thompson and Hamilton (1974) and Hobbie and Rublee (1977). Hobbie and Rublee conclude that it is caused by adsorption of ^{14}C label onto the filters, which, because filters have only a limited number of adsorption sites, adds a constant activity to a filter, irrespective of volume filtered. They suggest washing with unlabelled substrate. Because all filters were washed and moreover, a constant sample size of 10ml was used throughout my work, this problem was discounted. The second "filtration error" was noted by Albright and Wentworth (1976) who noted that recovery of ^{14}C activity on filters was

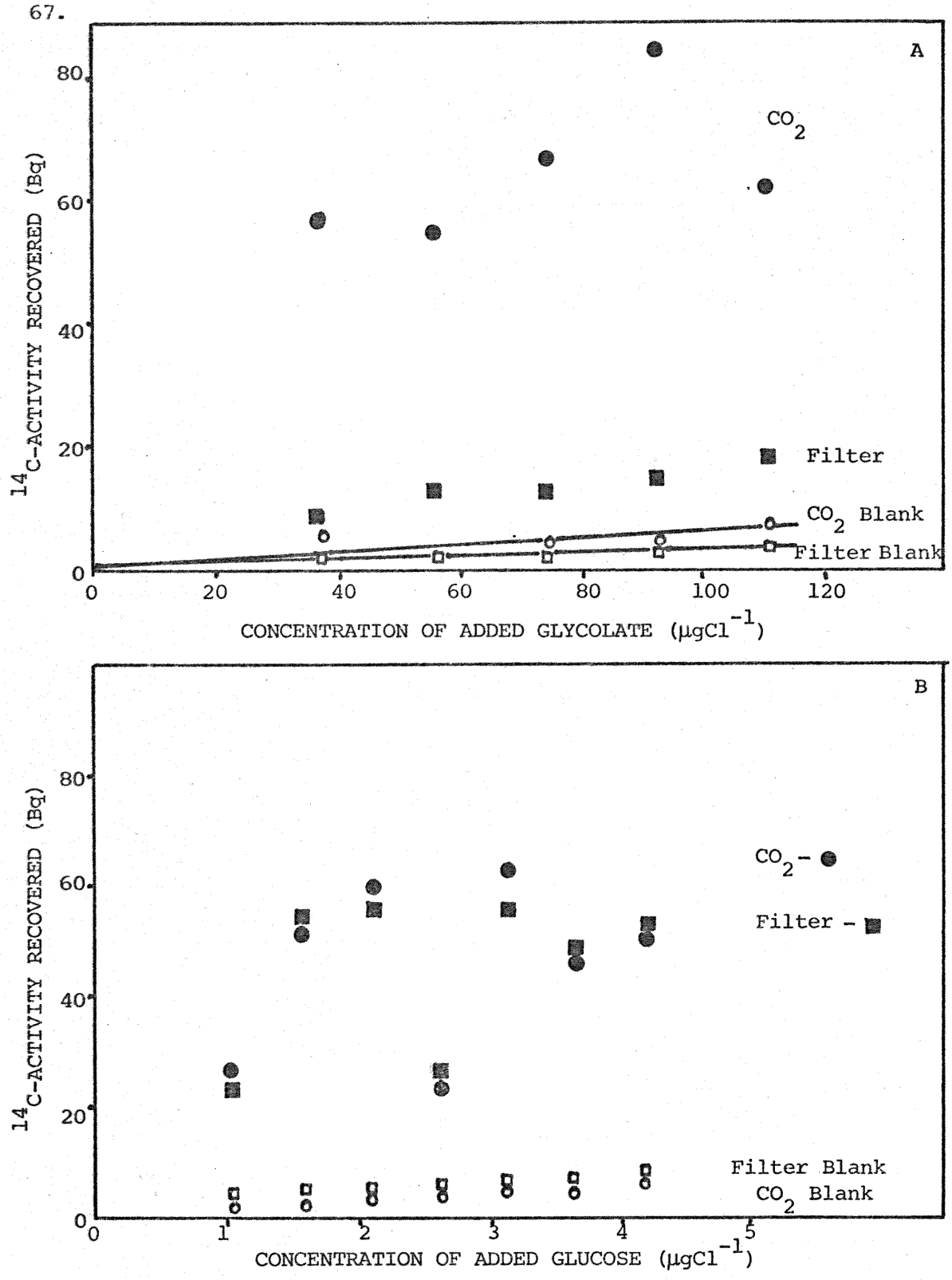


Figure 2.2/11 - The Relationship between Blank Values and ^{14}C-Recovery on Two Occasions

A - Lake Fryxell, 0030h on 4/1/81, glycolate uptake in dark

B - Lake Rotomanuka, 1400h on 3/4/81, glucose uptake

(see section 2.2.5)

also influenced by the volume of wash water used. For this reason, a constant wash volume of 10ml was used.

2.2.5 Blanks and Controls

Blanks were used to correct for (i) $^{14}CO_2$ in the substrate and possible abiological oxidation of substrate during incubation, and (ii) the adsorption of ^{14}C substrate on to detritus, cells and the membrane filter.

Blanks were prepared by incubating samples of lake water with a final concentration of 0.8% formaldehyde (0.2ml 40% formalin in 10ml) for at least one hour prior to the addition of substrate. They were then incubated with substrate for a further 30-60 minutes. CO_2 recovery and filtration were carried out in the normal way. It was found, in preliminary experiments, that if formalin was added immediately prior to substrate addition the blanks were four to six times higher than if formalin was added one hour prior to substrate addition. Because of the uncertainty of the relationship between abiological adsorption processes and substrate concentrations, a blank was obtained for each substrate concentration. This is recommended by Thompson and Hamilton (1974). In fact, various relationships were found between filter blank value and substrate concentration at different times. This was only true of the filter blanks though, as the CO_2 blanks were all related to residual $^{14}CO_2$ in the substrate solution. This fact was determined by recovering $^{14}CO_2$ from substrate added to 10ml aliquots of sterile Milli-Q reagent grade water.

Where the relationship between blank value and substrate concentration was not clear because of the errors associated with such low levels of activity, a linear relationship was assumed and blank values at each concentration were calculated using a linear regression based on all values.

Blank values were determined once for each 24 hour period and once at the middle of the five day investigation of Lake Fryxell.

To give some idea of the relationship between blank value and ^{14}C-uptake, this is shown over a range of concentrations of added substrate, for each substrate, in figure 2.2/11.

2.2.6 Liquid Scintillation Counting

Scintillation counting of ^{14}C-activity depends on the existence of scintillators, organic compounds which absorb the energy of β-particles emitted by decaying ^{14}C-atoms and re-emit some of this energy as photons of light in the blue end of the visible spectrum. (The radioactive decay of the ^{14}C-isotope involves the release of one beta-particle per ^{14}C-atom.) These photons have a range far in excess of the ^{14}C beta-particle (which is less than 3mm in water) and can be detected at some distance from their source by a photomultiplier. Scintillators, dissolved in suitable solvents, such as toluene, provide the basis for liquid scintillation counting in which by dissolution of labelled sample in the same solvent ("cocktail"), an intimate contact is achieved between ^{14}C and scintillator. Pulses of photons are released at each ^{14}C decay. These vary in photon number and hence energy, according to the energy of the liberated beta-particle and to the extent of interference (quenching) in the processes occurring between ^{14}C decay and the arrival of light at the photomultiplier. Plotting pulse number against pulse energy produces an energy spectrum characteristic of the isotope, cocktail and extent of quenching. Changes in quenching are caused by the presence in samples of quenching substances; substances which interfere with photon production or photon release from the cocktail. See reviews of Kobayashi and Maudsley (1974) or Hash (1972).

Scintillation counting was done in a Packard Tri-Carb (Model 3330) Liquid Scintillation Spectrometer. Scintillation vials were 20ml glass with plastic screw tops and removable conical Poly Seal cap liners (Wheaton Scientific, Millville, New Jersey, U.S.A.).

It was desirable for simplicity to use only one type of cocktail. This cocktail required the following attributes:

(i) Ability to incorporate up to 300 µl of an aqueous solution

(ii) Ability to incorporate up to 300 µl of the 2-phenylethylamine : 2-ethoxy-ethanol trapping mixture

(iii) Ability to dissolve a wet membrane filter. Because neither the opacity of an undissolved membrane filter nor the extent to which ^{14}C labelled cells would be released from the filter in a cocktail was known, it was felt that it would be desirable to dissolve the membrane filters used for harvesting labelled bacteria, in the cocktail. This would negate any effect that

the geometry of the filter and attached bacteria, in the scintillation vial, might have on counting efficiency. Because of the finding of Wallen and Green (1968) that ^{14}C-activity can be lost from cells dried on membrane filters it was felt to be necessary to use a cccktail which would dissolve a wet membrane filter.

Kobayashi and Maudsley (1974) suggested a mixture of toluene and ethanol or 2-ethoxy-ethanol as a general purpose cocktail solvent. Pugh (1973) suggested a toluene-2-methoxy ethanol mixture as a cocktail solvent for counting wet membrane filters from primary productivity studies. After some initial experimentation the following mixture was chosen, on the basis of cost and because it fulfilled the above requirements.

Each litre contained
700ml toluene (solvent grade)
200ml absolute ethanol (solvent grade)
100ml 2-ethoxy-ethanol (scintillation grade, BDH Chemicals Ltd, Poole, England)
6g 2,5-diphenyloxazole (PPO) (Sigma Chemical Company, St. Louis, Missouri, U.S.A.), primary scintillator.

The maximum counting efficiency of this cocktail was measured using standardised ^{14}C n-hexadecane of 28,000 Bq g^{-1} (The Radiochemical Centre, Amersham) in 10ml of cocktail. This was 53.5% ± 1.0% (mean ± standard deviation n=3). No improvement in counting efficiency could be had by using AR grade toluene (BDH) or by using AR ethanol (BDH). AR 2-ethoxyethanol raised the efficiency to 58.9% ± 1.0%. Pugh used a secondary scintillator, supposedly to improve counting efficiency, but as his reported counting efficiency was only 55%, a secondary scintillator was not used here. The optimum concentration of PPO was determined experimentally. Below 6g l^{-1} counting efficiency decreased. Above 6g l^{-1} it did not increase. For each sample 10ml of cocktail was dispensed from a reagent dispenser.

For the samples from Lake Fryxell which contained 1.5ml of trapping mixture (1:2, v:v 2-phenylethylamine:2-ethoxyethanol) a different cocktail was used. This consisted of 8.5ml per sample of toluene containing 6g l^{-1} PPO. This, mixed with 1.5ml of trapping mixture gave a counting efficiency of 54.0% ± 0.8%.

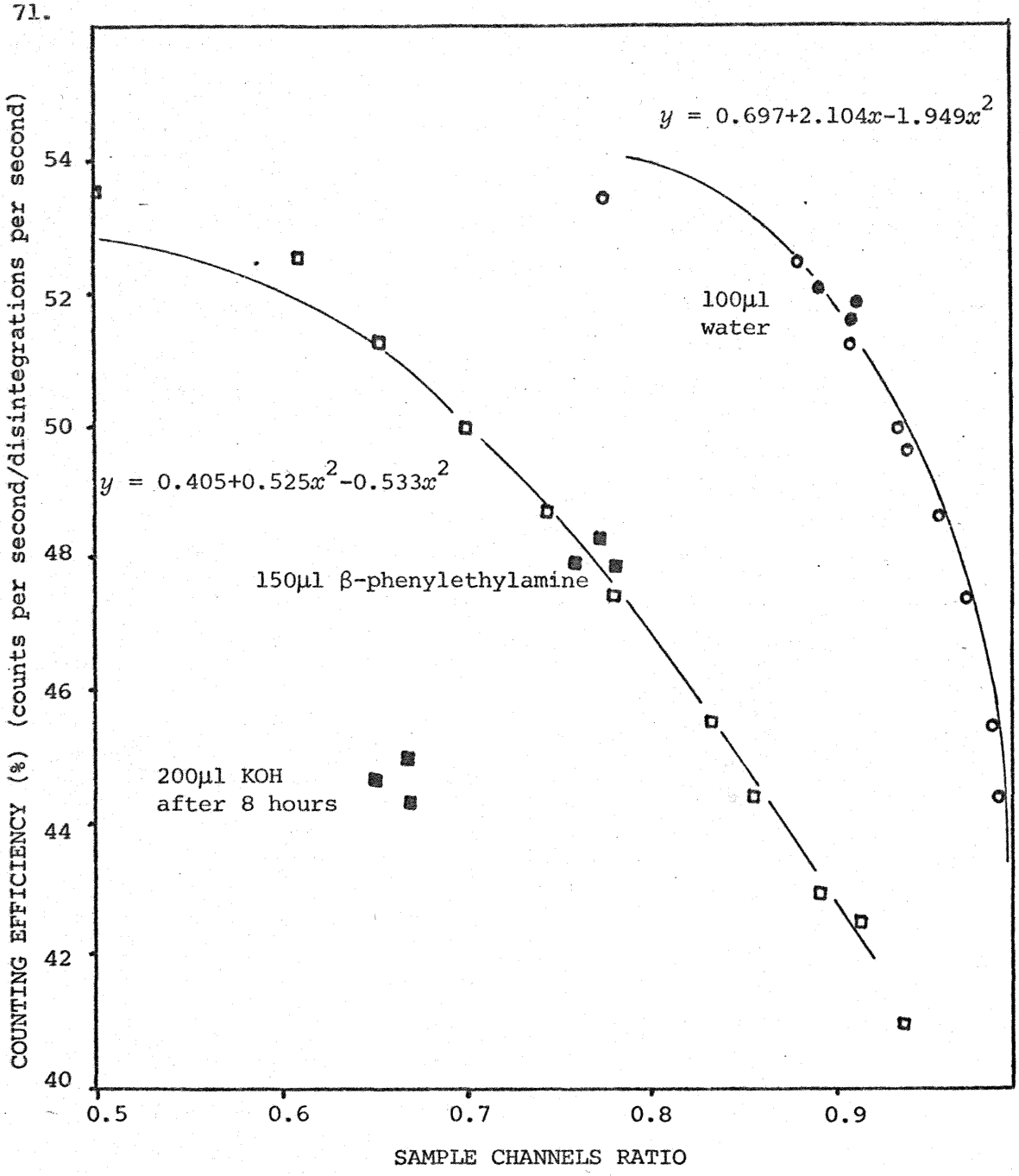


Figure 2.2/12 - Quench Curves used for Determining Counting Efficiency using the Sample Channels Ratio Method

Open circles depict the effect on both counting efficiency and ratio of counts in Green channel (width 50-500) to counts in Red channel (width 50-1000), of repeated addition of drops of acetone.

Open squares show the similar relationship between counting efficiency and the ratio of counts in the Blue channel (width 50-300) to counts in the Red channel (width 50-1000)

Solid lines are fitted curves

Solid circles show the quenching effect of 100µl water
Solid squares show the quenching effect of 150µl of β-phenylethylamine or 200µl of KOH

Quench corrections were made using the sample channels ratio (scr) method of Bush (1963). The effect of quenching is to remove photons from the photon pulses caused by ^{14}C decay, thus altering pulse energy and making some pulses too feeble to be distinguishable from background, resulting in decreased counts per second. In terms of the pulse energy spectrum which is unimodal in homogeneous samples, increased quenching causes a shift in the peak so that maximum pulse numbers are found at a progressively lower pulse energy. The scr method uses two separate counting channels, one which counts total pulses per second which are above background energy and the other which counts only a portion of the pulse energy spectrum peak. As this peak shifts, there is a measurable relationship between the ratio of the counts in each channel and the total number of counts in the broad counting channel, i.e. between channels ratio and counting efficiency. This relationship is dependant on the scintillation cocktail used and can be determined by repeatedly adding aliquots of a known quenching substance to cocktail containing a known ^{14}C activity and counting in each channel between additions. On the suggestion of Kobayashi and Maudsley, acetone was used as a quenching agent and 1 or 2 drop aliquots were added with a Pasteur pipette. The machine settings and background counts in each channel with the cocktail used were as follows:

Channel	Red	Green	Blue
Gain	23%	23%	23%
Channel width (arbitrary units)	50-1000	50-500	50-300
Background (counts per second ± standard deviation)	0.70 ± 0.31	0.57 ± 0.25	0.45 ± 0.22

For the red channel which accumulated total counts, gain was set to give maximum counts per second for an unquenched sample containing ^{14}C n-hexadesane. The channel width, which determines the portion of the pulse energy spectrum from which pulses are counted, was set to give minimum background counts. The other two channels were each used for a separate range of counting efficiencies, the green channel from 54% to 47.3% and the blue channel from 47.3% to 43.0%. In each of these channels, over its particular range of use, counts were never less than 75% of total counts. Thus, counting accuracy in these channels was only slightly less than in the red channel. The relationships between channel ratios and counting efficiency are shown in figure 2.2/12, along with the quenching effect of various sample types.

The curves in figure 2.2/12 were fitted using a least squares quadratic regression programme on a Hewlett Packard HP9100A programmable calculator. Based on the equations for these relationships a program was written for the HP9100A which would calculate sample activity in Bequerels (disintegrations per second) from counting time and the counts accumulated in each channel. This program also corrected for the background counts in each channel. These were determined by counting 30 replicate aliquots of cocktail, with no added activity, in 30 vials selected at random from vials which had been used already and washed as per the method given in section 2.2.7. The same program was used to correct for quenching in the toluene-only cocktail used for Fryxell $^{14}CO_2$ samples.

The effect of dilution on counting efficiency, by samples or by the acetone used for constructing quench curves, was found by addition of toluene to samples, to be insignificant between 10ml and 11ml total volume.

All samples and standards were counted to a total of at least 10,000 counts in the red channel giving a standard counting error of 1%, which was insignificant in comparison with other errors in the heterotrophic uptake technique as a whole.

2.2.7 Washing and Waste Disposal

All equipment which came into contact with ^{14}C solutions, except for scintillation vials and lids, was washed as follows.

(i) Immersed in a 2-3% Decon 90 (Decon Laboratories, Brighton, England) solution for at least 12 hours followed by scrubbing, if this was necessary to remove solid residue.

(ii) Immersed in tap water overnight followed by thorough rinsing under running tap water. In Antarctica, soaking and agitation of incubation vessels and septum seals in one container of rinse water was all that was done in this respect.

(iii) Rinsed in distilled water. In Antarctica glacial ice was melted, filtered (Whatman GF/C), and used for this purpose.

(iv) Dried in a warm drying oven, or in the open.

Syringes, septum seals and blocked hypodermic needles were re-used without sterilisation. The glass tubes used in the CO_2 trapping chimneys were pre-soaked in hot 5% Decon 90.

Scintillation vials were emptied in a fume cupboard, the liners were removed from the caps and vials, caps and liners were given a rinse in methanol to remove most of the water insoluble cocktail. They were then soaked at least 12 hours in (initially) hot 2-3% Decon 90 and treated as per steps (ii), (iii) and (iv) above.

Aqueous waste was disposed of by dilution into the general waste water system at ^{14}C-concentrations and at times in accordance with the "New Zealand Code of Safe Practice for the Use of Unsealed Radioactive Materials" (National Radiation Laboratory - 1971).
This includes all aqueous waste generated in Antarctica by incubation and washing, which was returned to New Zealand in accordance with Antarctic Treaty Regulations.

Solid waste and used cocktail were stored until disposal by the Radiation Safety Officer.

2.2.8 Data Processing and Error Limits

After correction for efficiency of liquid scintillation counting and efficiency of recovery of CO_2, data was expressed as t/f, where t was the incubation time and f was the fraction of added ^{14}C-activity which was subsequently recovered as either ^{14}C-biomass or $^{14}CO_2$. Data processing to this stage was performed by a computer programme written in Fortran by C.H.W. Sitwell of the University of Waikato Computer Services department.

The kinetic parameters, V_{max}, $(K_T + S_n)$ and T_t were calculated with the aid of the multiple linear regression programme (one independant variable) of a computer software package (B.M.D.P., Software Development Inc., U.S.A.) available on the University of Waikato computer system. This programme provided the equation describing a linear relationship between t/f and A, the concentration of added substrate (both ^{14}C-labelled and unlabelled). As discussed in section 1.2.3, T_t is equal to t/f when A is equal to zero, V_{max} is equal to the reciprocal of the slope of the relationship between t/f and A (where A is the independent variable) and $(K_T + S_n)$ is equal to T_t multiplied by V_{max}. (See equation 9 and figure 1/1B, section 1.2.3).

The above mentioned programme also calculated r, the Pearson's product moment correlation coefficient, which quantifies the 'goodness of fit' of the data points to the calculated line of best fit, and P, the probability that the slope of the assumed linear relationship between t/f and A is equal to zero (i.e. no relationship or a non-linear relationship). (See Sokal and Rohlf (1973) for further discussion of the calculation and interpretation of r and P). Gillespie and Spencer (1980) used a value of P of less than 5% as a condition for the acceptance of a set of data points from a heterotrophic uptake experiment. This approach is useful for eliminating data sets which will yield kinetic parameters which are not significantly different from zero when the absolute value of kinetic parameters is of interest. However, it was felt to be too restrictive a criterion for the acceptance of data sets for a study of changes in heterotrophic uptake kinetics. This was because a situation might arise in which the results from one heterotrophic uptake experiment might not be acceptable (using the criterion that $P<0.05$) but might be significantly different from the acceptable results of another heterotrophic uptake experiment. In view of this it was necessary to calculate the 95% confidence limits of each of the kinetic parameters, V_{max}, T_t and $(K_T + S_n)$, from the results of each experiment, to enable a comparison to be made between the values calculated for each parameter from the results of different experiments. Different values of the same parameter which had overlapping 95% confidence limits were considered to be not significantly different. Values with 95% confidence limits which did not overlap were considered to be significantly different.

The 95% confidence limits of each parameter were calculated as follows. For the sake of simplicity, in each of the following examples, a linear relationship based on n data points (x_i, y_i) is considered. This relationship is of the form $y = bx + a$, where y is the dependent variable, x is the independent variable, b is the estimated slope of linear relationship and a is the estimated y-intercept.

(a) The 95% Confidence Limits of V_{max}

$$V_{max} = b \pm t^{.05}_{n-2} \times \frac{\sqrt{\frac{\Sigma (a+bx_i-y_i)^2}{n-2}}}{\sqrt{\Sigma(x_i-\bar{x})^2}} \times \frac{1}{\quad} \qquad \text{equation 1}$$

where $\bar{x}$ is the mean of the x-variables, and

$t^{.05}_{n-2}$ is the Student's t value for a two-tailed test with a probability of 0.05 and n-2 degrees of freedom.

(b) The 95% Confidence Limits of T_t

$$T_t = a \pm t^{.05}_{n-2} \sqrt{s^2_{y.x}\left[\frac{1}{n} + \frac{(x-\bar{x})^2}{\Sigma x_i^2}\right]} \qquad \text{equation 2}$$

where $t^{.05}_{n-2}$ and $\bar{x}$ are as in (a) above, x in this case is equal to zero,

and $s^2_{y.x} = \frac{\Sigma(a+bx_i-y_i)^2}{n-2}$. equation 3

(see Sokal and Rohlf (1973)).

(c) The 95% Confidence Limits of $(K_T + S_n)$

$(K_T + S_n) = \frac{a}{b}$ with 95% confidence limits of $-(\bar{x}-d_2, \bar{x}-d_1)$

where d_1 and d_2 are the two roots of the quadratic equation.

$$d^2\left[b^2 - \frac{s^2 F^{.05}_{1,n-2}}{\Sigma(x_i-\bar{x})^2}\right] - 2d\bar{y}b + \left[\bar{y}^2 - \frac{1}{n} s^2 F^{.05}_{1,n-2}\right] = 0 \qquad \text{equation 4}$$

where $s^2 = \frac{n-1}{n-2} s^2_y \quad (1-r^2)$

s^2_y = the variance of y values

$\bar{x}$ = the mean of x values

$\bar{y}$ = the mean of y values

r = the correlation coefficient of the correlation between x and y

$F^{.05}_{1,n-2}$ = the F value with 1 and n-2 degrees of freedom which is exceeded with a probability of 5%.

This method for calculating the confidence limits of an x-intercept is from Snedecor and Cochran (1976) who point out that it will only work where the coefficient of d^2, in the quadratic equation, is positive (that is, if the line is not too flat).

The roots of the quadratic equation ($Ad^2 + Bd + C = 0$) were found by solving the following formula:

$$d_1, d_2 = \frac{B}{-2A} \pm \frac{1}{2A} \sqrt{B^2-4AC} \qquad \text{equation 5}$$

using a Hewlett Packard 9100A programmable calculator and a programme supplied by the manufacturer.

2.3 DIRECT COUNTING OF BACTERIA BY EPIFLUORESCENT MICROSCOPY

Because of the doubtful relevance of the traditional microbiological technique of plate counting when applied to environments such as lakes, which are impoverished in nutrients relative to most microbiological media, direct counting is becoming a favoured technique for the enumeration of bacterial numbers in aquatic environments. This trend has been encouraged by the introduction of biologically selective fluorescent dyes, such as acridine orange which binds to nucleic acids and fluoresces when exposed to ultraviolet light. Cells stained with acridine orange and illuminated with ultraviolet light from above, appear fluorescent against a black background, a feature which significantly improves their resolution compared with that obtainable with traditional staining techniques.

From an examination of the literature it is apparent that there are almost as many acridine orange staining techniques as there are authors. For examples see Zimmermann, Iturriaga and Becker (1978), Ramsay (1974) and Meyer Reil (1978). From reviews on methodology (see Daley and Hobbie, 1975, and Jones and Simon, 1975), it is equally apparent that there is no one best technique, as yet, and that as long as consideration is given to certain factors which are known to affect results, uniform application of technique is probably more important than the technique itself. The following is the sequence of steps used in the technique which was adopted here. It is followed by discussion of some of the significant aspects of each step.

(a) Sample Collection and Storage

Water samples were collected in new 10ml vacutainers (Becton, Dickinson and Company, Rutherford, N.J., U.S.A.) or in universal bottles which had been thoroughly washed then rinsed in filtered (0.2μm) distilled water and dried. Samples were fixed in a final concentration of 0.8-1.0% formaldehyde and stored, as soon as possible after collection, in the dark at 0.4°C.

(b) Sample Dispersal

Dispersion was achieved by ultrasonication for 30 seconds at at 10.5 watts (8,000 watts cm^{-2}) in 5ml of sample plus buffer using a Kontes Micro-Ultrasonic Cell Disruptor (Kontes Glass

Company, Vineland, N.J., U.S.A.). Samples were held in an ice bath during sonication to prevent overheating.

(c) Filter Preparation

Membrane filters (0.2µm pore size, 25mm diameter Nuclepore polycarbonate membrane) were prestained, in 67 mgl^{-1} Sudan Black-B (BDH) in 50:50, v/v, distilled water/ethanol in order to reduce background fluorescence. Staining lasted for at least two hours. Filters were removed from the stain and placed directly in 16mm diameter glass filter funnel assemblies (Millipore) and 2ml of 0.02% Tween 80 (Becton, Dickinson and Co.) in distilled water was run through each filter under vacuum to remove excess stain and to improve uniformity of bacterial distribution on the filters by increasing filter wettability.

(d) Sample Filtration

An appropriate volume of sample from 0.1ml to 1.0ml was made up to 5ml with 0.1M phosphate buffer (pH 8.2) and filtered.

(e) Staining and Destaining

The following solutions were added to the filter funnel for the stated lengths of time and were then removed by application of vacuum.

(i) 2ml of acridine orange stain (Sigma practical grade) at 0.4 mgl^{-1} in 0.1M phosphate buffer, pH 8.2, two minutes;
(ii) 2ml 0.1M phosphate buffer, pH 7.2, run straight through;
(iii) 2ml 0.1M phosphate buffer, pH 4.6, run straight through.

(f) Filter Mounting

While still damp, filters were layered, carefully to exclude air bubbles, on to chromic acid-washed glass microscope slides. Immediately, a small drop of Cargille A immersion oil (R.P. Cargille Laboratories Inc., Cedar Grove, N.J., U.S.A.) was placed on each filter and covered with a cover slip.

(g) Observation

Observation followed within two hours. The microscope used was a Leitz Ortholux II (Ernst Leitz Wetzlar Ltd, West Germany) fitted with a high pressure mercury lamp (Osram HBO 220W/4),

a Fluortar X100 objective lens and a X12.5 eye piece lens (total magnification X1250). A BG38 filter was fitted to the light source, a K510 barrier filter was used in the eyepiece and TK510 and K515 dichroic beam splitter filters were used. Random fields were counted until at least 400 individual bacterial cells had been counted in each sample.

Discussion of Technique

All reagents used, except for absolute ethanol, were filtered through a 0.22μm membrane filter (Millipore GS) prior to use. For this purpose a syringe with attached filter holder was used. Additionally, all buffers were preserved with 2% added formaldehyde prior to filtration. Filtration apparatus was cleaned between samples in 70% ethanol followed by a rinse in freshly filtered (0.2μm) distilled water.

(a) The fixation and storage methods used were those of Zimmermann, Iturriaga and Becker (1978), who observed no loss of formalin-fixed cells after one month. Maximum storage of the samples was six weeks for those from Lake Fryxell.

(b) Dispersal was particularly important for Lake Fryxell samples which were clumped so as to preclude meaningful counts. Because clumping was not observed in fresh samples, using phase microscopy, it was assumed that clumping was an effect of storage, possibly due to formaldehyde crosslinking. In samples from Lake Rotomanuka, bacteria were often associated with detrital particles, necessitating dispersal. Sonication conditions were determined empirically, the conditions described giving satisfactory results. Shorter times and lower power settings did not achieve full dispersal. No attempt was made to determine the effect of sonication on cell numbers because of the difficulty of counting clumped cells. Work by Cooper (*pers. comm.*) on pure cultures of aquatic bacteria suggests that the sonication treatment used here was unlikely to result in significant loss of cells.

(c) The procedure for staining filters was adopted from Zimmermann, Iturriaga and Becker (1978). Because Sudan Black precipitates from 50% ethanol after only a few days, the stain was made up fresh each time it was needed by adding filtered (0.2μm)

distilled water to a solution of 134 mg of Sudan Black in one litre of absolute ethanol. The stain was not filtered because of the disruptive effect that 50% ethanol has on the mixed cellulose ester filters used (Millipore GS).

Polycarbonate filters were chosen because of their flat surfaces and uniform pore sizes which result in all filtered bacteria ending up in essentially the same focal plane (Daley and Hobbie, 1975). Non-uniform wetting of polycarbonate filters is a major problem affecting their use for direct counting (Jones and Simon, 1975). It results in patchy distribution of bacteria across the filter surface. On the filters used here, the sizes of wetted and unwetted patches were in the order of 50-300μm across. The use of 0.02% Tween 80 eliminated patchy distribution. 0.2% Tween 80 was equally effective but removed the Sudan Black stain. As an example of the effectiveness of pretreatment of filters with Tween 80 the mean counts per field ± one standard deviation on an untreated filter were 71 ± 31 (18 fields) and for the same sample on a treated filter, 93 ± 9 (12 fields).

(d) The choice of a 5ml volume for filtration was based on the findings of Jones and Simon (1975) that smaller volumes results in a disproportionate number of bacteria being concentrated at the filter periphery.

(e) The choice of 0.1M phosphate buffers was based on their use by Cooper (*pers. comm.*). Zimmermann, Iturriaga and Becker (1978) mention the beneficial effect on cell resolution against background fluorescence, of staining at a slightly alkaline pH and destaining at an acid pH.

(f) The importance of not drying filters prior to observation is covered by Jones and Simon (1975). Drying results in decreased cellular fluorescence and consequently decreased cell counts. Damp, but not wet, filters could be layered on to glass slides in such a way that they were completely flat and immobile. Cargille A immersion oil is recommended by Zimmermann *et al* as highly suitable for epifluorescent microscopy.

Chromic acid washing of slides was used to remove a surface film, found on some slides, which caused severe background fluorescence. This problem was not noticed with cover slips.

(g) The microscope lamp and filter combination was selected empirically from available combinations. Criteria used for selection were (i) minimisation of background fluorescence, (ii) minimisation of fading of fluorescence of cells and (iii) maximisation of cell brightness.

Mean cells per field were converted to cells per mililitre in the original sample by the following calculation:

$$\text{cells per field} \times \left[\frac{804\text{mm}^2 \text{ (filtration area)}}{0.0081\text{mm}^2 \text{ (field area)}} \times \frac{10.5\text{ml (sample + formalin)}}{10\text{ml (sample)}} \times \frac{1\text{ml}}{x\text{ml}} \right] = \text{cells per ml}$$

Where x = sample volume filtered.

Assuming a perfectly random distribution of bacteria on the filter and a total of 400 cells counted in each sample, the standard error for each count could be calculated, using the Poisson distribution, as $\sqrt{400}$ or 5% of the total number of cells counted. From this standard error 95% confidence limits were calculated as cells per ml ± 10% (10% is approximately equal to 1.96 times 5%). (See Sokal and Rohlf, 1973).

2.4 PHYTOPLANKTON BIOMASS ESTIMATION BY EXTRACTION OF CHLOROPHYLL *a* AND PHAEOPHYTIN

Ideally, phytoplankton biomass estimation would have involved direct counts of algal cells with subdivision at the genera level. Because of the time-consuming nature of this technique a simple pigment extraction technique was used (Vollenveider, 1969). From 300ml to 1,000ml of lake water was filtered through a 55mm Whatman GF/C filter disc (nominal pore size 1.0μm) in a glass Millipore filter funnel. One ml of 1.0% $MgCO_3$ suspended in distilled water was added to each sample prior to filtration. In the field a hand vacuum pump was used and vacuum was kept at less than 13 kPa to minimise damage to cells. Some examples of literature values for maximum pressure differentials range from 6.5 kPa (Hellebust, 1968) to 26 kPa (Williams and Yentsch, 1976) with intermediate values of 13 kPa (Zimmerman, 1977) and 20 kPa (Salonen, 1974). The reason given for maintaining such low differential pressures is in each case to minimise cellular damage but none of the above authors explain why a particular differential pressure value is chosen. A pressure differential of 13 kPa was chosen purely because it was in the middle of the quoted range. The filter disc was trimmed down to the area actually used for filtration and was stored, sealed in a test tube containing 2ml of methanol (BDH - AR grade containing 0.1% $MgCO_3$ to neutralise).

In the laboratory each tube was unsealed and heated in a boiling water bath for 30 seconds. After cooling the filter was removed and the methanol topped up to 2ml using marks previously inscribed on the test tube. Methanol extracts were centrifuged to remove particulate matter (5,000g for 10 minutes in a Sorval RC2B centrifuge (Du Pont Company, Wilmington, U.S.A.) at 4°C for samples from Rotomanuka and 4,500g for 10 minutes in an MSE Super Minor (Measuring and Scientific Equipment Ltd, Crawley, Sussex, England) at -5°C to +5°C at Lake Fryxell).

Absorbances were read at 665nm, the chlorophyll *a* absorption maximum, and at 750nm, to correct for particulate interference. Instruments used were a Beckman 24 double beam spectrophotometer (Beckman, U.S.A.), for samples from Lake Rotomanuka and a Spectronic 20 (Bausch and Lomb, U.S.A.) at Lake Fryxell. The two machines gave identical results at

665nm and 750nm when the same sample was analysed in both. The result was expressed as:

$$\text{Chlorophyll } a \ (\mu g l^{-1}) = 13.9 \ (O.D._{665} - O.D._{750}) \times \frac{\text{extract volume}}{\text{sample volume}}$$

No attempt was made to correct for phaeophytins, chlorophyll degradation products which also absorb at 665nm. The difficulty of doing so in methanol extracts is discussed by Marker (1977).

The technique used provided evidence for algal migration in Lake Fryxell where peaks of "chlorophyll" were seen to move in the profile (see section 4.3). In Lake Rotomanuka, on all but one occasion, samples were only taken from the 1m sampling depth used for heterotrophic uptake samples. In each case at 1m depth chlorophyll *a* decreased at night (see section 3.2). However, this may have been due to a decrease in unit chlorophyll per cell at night time; a common phenomemon in this lake (Pridmore, *pers. comm.*). Thus, the technique, as used, was not sufficient to provide convincing evidence for or against algal migration in Lake Rotomanuka. Sampling at more depths would have been necessary to follow algal migration patterns.

2.5 MEASUREMENT OF ELECTRON TRANSPORT SYSTEM ACTIVITY BY REDUCTION OF INT

Because a fluorescent microscope was not available at Lake Fryxell and because this lake was known to be finely stratified (Vincent, 1981), a technique was needed for determining the depths at which peaks of biological activity occurred and whether these peaks migrated at all. The technique chosen was one of the many which rely on the fact that 2-(*p*-iodophenyl)3(*p*-nitrophenyl)-5 phenyl tetrazolium chloride (INT) is reduced by accepting electrons from the flavoproteins, quinones or cytochromes of electron transport systems. The reduced INT-formazan is coloured, absorbing maximally at 490nm. (See Christensen and Packard (1978) for a review of methodology.) The specific technique used here was that of Jones and Simon (1979). As INT can accept electrons at the flavoprotein level of electron transport it has the potential to measure anaerobic as well as aerobic electron transport activity. Reports in the literature of positive results in anaerobic zones such as lake sediments support this view (Zimmerman, 1975 and Jones and Simon, 1979).

Previous work with INT reduction techniques has shown only partial correlation between measured electron transport system (ETS) activity and other measures of biomass, for example protein content (Riechardt, 1979), ATP analysis and bacterial direct counts by epifluorescent microscopy (Jones and Simon, 1979), and ATP analysis (Devol, Packard and Holm Hansen, 1976). Jones and Simon conclude that INT reduction techniques may be used to indicate sites of potential microbial activity but not to predict, with accuracy, their oxygen uptake or biomass content. However, as the ETS method of Jones and Simon is capable of yielding quantitative results from many samples within a few hours it was thought that it might provide a useful basis for the selection of heterotrophic uptake sampling depths in Lake Fryxell.

The following is a summary of the methodology; for a more thorough discussion see Jones and Simon (1979).

(a) Sample Collection and Filtration

Samples were collected from different depths as described in section 4.2. 300ml samples were returned to the laboratory in sealed polythene jars. 100ml aliquots from each depth were

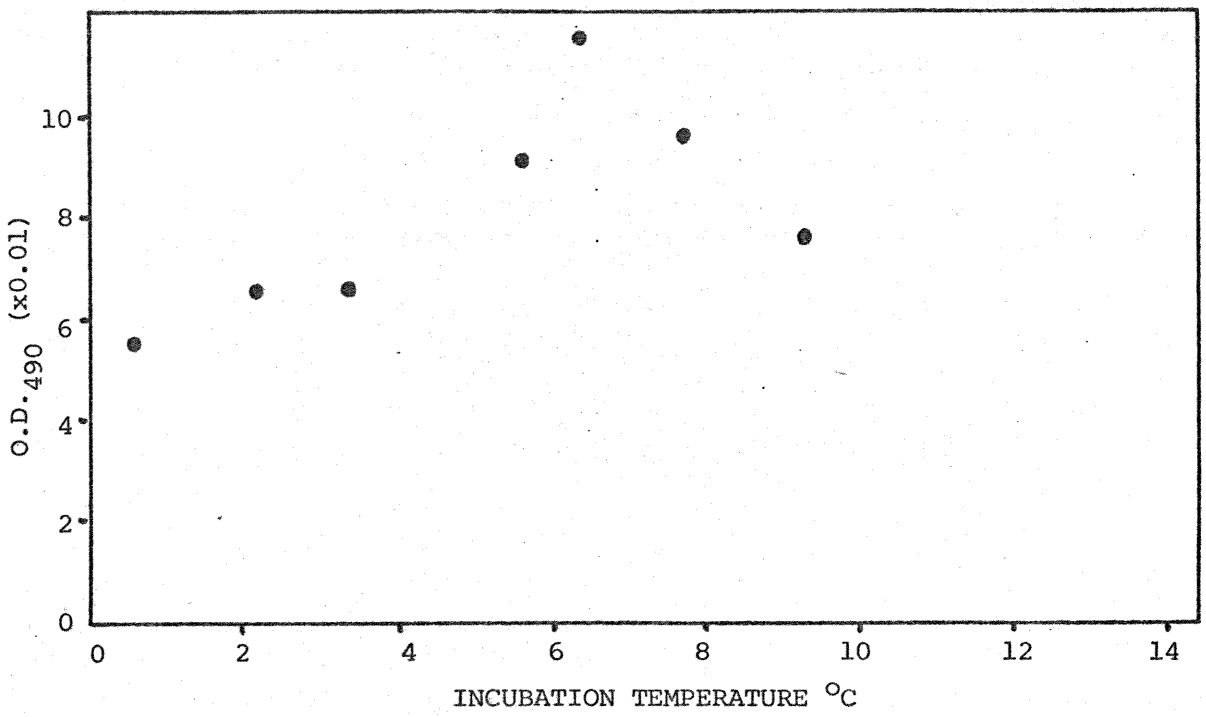


Figure 2.5/1 - The Effect of Incubation Temperature on Measured ETS Activity

30ml of water from 9.0m in Lake Fryxell filtered on to 0.45µm membrane filter and extracted as described in section 2.5(b). Assayed for one hour (section 2.5(c)). Assays run at same time. No replicates. Blank at each temperature.

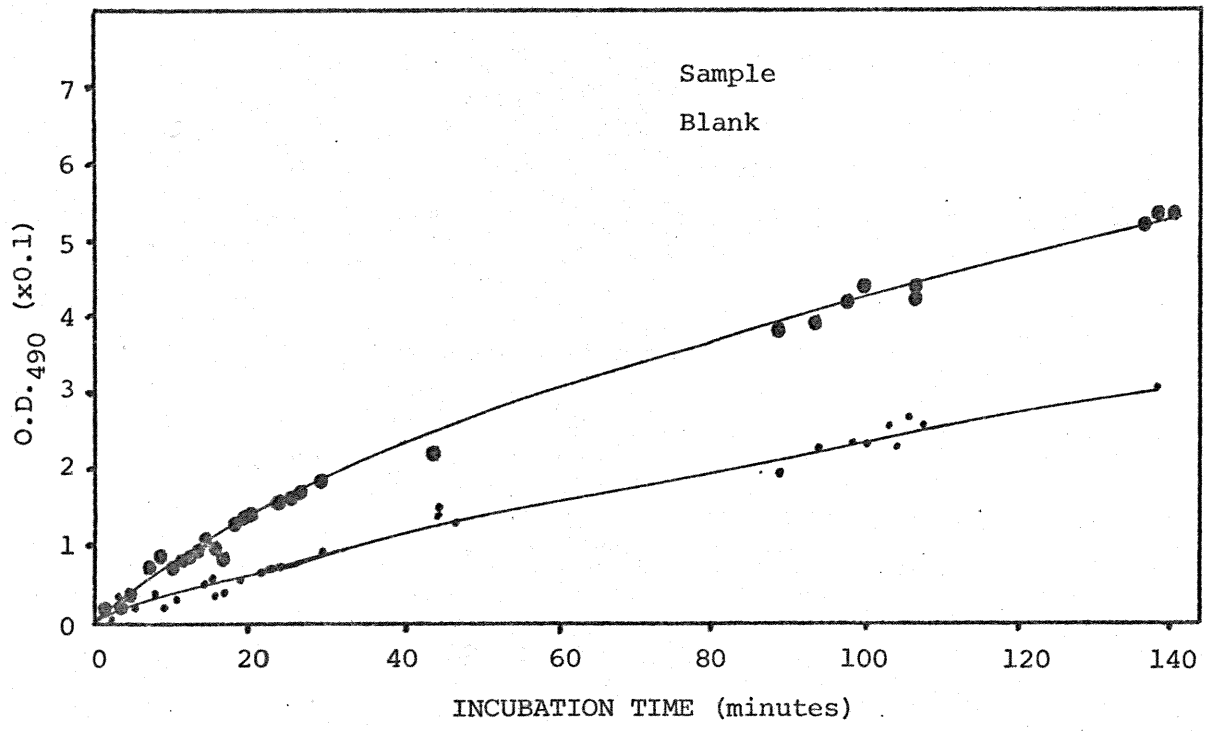


Figure 2.5/2 - Increase in INT-formazan Production with Time in Assay Containing Enzyme Extract (sample) or Boiled Enzyme Extract (blank)

Extract from 9.0m in Lake Fryxell. 30ml filtered on to 0.45µm membrane filter. Extracted as described in section 2.5(b). Assayed at 10 °C ± 0.5 °C

filtered on to 25mm diameter discs of glass fibre filter paper (Whatman GF/C, nominal pore size, 1.0µm). An initial attempt at using 0.45µm membrane filters (Millipore HA) resulted in three times higher ETS activity per sample but membrane filters were not subsequently used because of their extremely slow filtration time. Only one filter column was available and each 25ml sample took 20 minutes to filter through a .45µm membrane filter. This was because of the high level of suspended solids in Lake Fryxell.

(b) Enzyme Extraction

Each filter was trimmed to remove the unused periphery and stored in 1.0ml of ice cold extraction buffer (for composition, see below). After all samples had been filtered each filter was sonicated in the extraction buffer for two minutes using a Kontes Ultrasonic Cell Disruptor set at maximum power (10.5W @ 8,000 Wcm^{-2}). Filter remnants were removed and extracts were centrifuged at 4,500g for 15 minutes in an MSE Super Minor centrifuge, at ambient temperature, which was generally in the range from -5°C to +5°C.

(c) Enzyme Assay

Assays were carried out at 7.5°C ± .5°C in a polystyrene encased water bath. Optimum temperature was determined for a single sample from 9m in Lake Fryxell (see figure 2.5/1). Each extract was assayed in duplicate. Assays were started by adding, with shaking, 40 µl of INT solution to 10 x 75mm test tubes containing 40 µl of enzyme extract and 120 µl of substrate solution. Assays were started at precise intervals and stopped at precise intervals, by the addition of 40 µl of termination solution to each tube, with shaking. The contents of each tube were transferred by Pasteur pipette to micro cells (Bausch and Lomb) for O.D. determination at 490nm in a Spectronic 20 single beam spectrophotometer. Water was used to zero the machine. Reference samples consisted of assays using enzyme extract, from at least four depths, which had been boiled for one minute.

In an initial experiment the time course of INT-formazan production was followed in assays of an enzyme extract and a

boiled extract from 9m depth in Lake Fryxell. At intervals the contents of each assay tube were removed and transferred to a micro cell for O.D. determination then returned to the assay tube. See figure 2.5/2 for results. Subsequent assays were limited to 30-40 minutes, during which time increase in $O.D._{490}$ varied substantially linearly with time.

(d) Composition of Reagents

(i) Extraction Buffer.

Triton X-100 (Sigma)	17 ml/L
Polyvinyl pyrrolidone (BDH)	$1.5 g l^{-1}$
$MgSO_4$	75 μmol l^{-1}
Tris buffer (Sigma)	50 mmol l^{-1}
EDTA (BDH)	5 mmol l^{-1}

Dry ingredients were weighed out in New Zealand. Triton X-100 and water were added at Lake Fryxell and the buffer pH adjusted to pH 7.4. Buffer was then distributed into universal bottles and sterilised in a pressure cooker.

(ii) INT Solution.

INT (Sigma, Grade I)	2mg ml^{-1}

(iii) Substrate Solution

Sodium succinate (BDH)	133 mmol l^{-1}
NADPH (Sigma, Type I, tetrasodium salt)	0.5 mmol l^{-1}
NADH (Sigma, Grade III, disodium salt)	3.4 mmol l^{-1}
Tris Buffer (Sigma)	50 mmol l^{-1}
EDTA (BDH)	5 mmol l^{-1}

In preparing both the INT solution and the substrate solution, quantities of the dry chemicals, apart from Tris buffer and EDTA, sufficient for 30 assays were weighed into dry glass bijou bottles, dehydrated over silica gel in an evacuated dessicator for five hours, sealed and stored in sealed plastic jars with dehydrated silica gel. The keeping properties of damp NADH are limited (Sigma Chemical Company. Warning with reagents). 25ml lots of Tris/EDTA buffer were made up adjusted to pH 7.4, and sterilised by autoclaving. At Lake Fryxell, INT solution and substrate solution were made up immediately prior to use by addition of water or Tris/EDTA buffer, respectively.

(iv) Termination solution. Equal parts by volume of 1.0M sodium formate (pH 3.5) and 40% formaldehyde.

(e) Expression of Results

Results are calculated as the sample O.D. minus the mean reference (blank) O.D. and are expressed as oxygen equivalents ($\mu g\ l^{-1} hr^{-1}$). A lower limit of detection for each assay is calculated from:

$$\text{lower limit of detection} = 2\sqrt{2}.t.S_b$$

where S_b is the standard deviation of the reference values and t = the Students t-value for a probability of 0.05 with a 1-tailed test with n-1 degrees of freedom (Jones and Simon, 1979).

(f) A Note on Blanks

Reference (blank) values were determined at different depths on six occasions. There was no indication of any relationship between blank value and depth, as might be the case if reduced compounds in the anoxic zone can reduce INT. Thus, a mean blank value used for assays from all depths was felt to be adequate. However, boiling the reference samples of enzyme extract would almost certainly oxidise any reduced compounds in these samples. For this reason, the values I have obtained as results from this technique are not corrected for chemical reduction. Reichardt (1979) discusses the difficulty of inhibiting enzyme activity without inhibiting chemical reduction and concludes that a suitable reference technique for the ETS method is lacking.

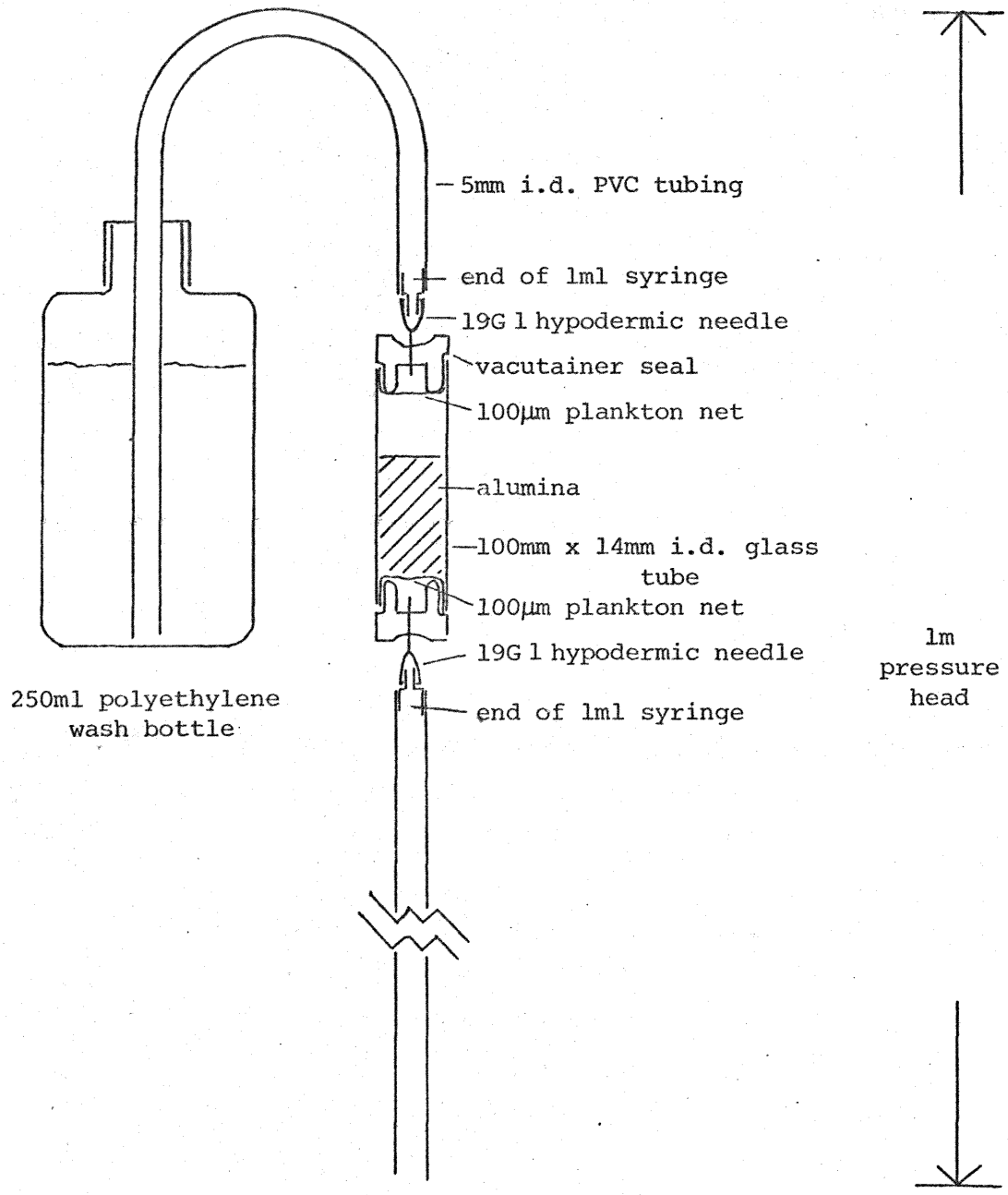


Figure 2.6/1 - Column used for Glycolate Absorption on to Alumina

(see section 2.6(a))

2.6 GLYCOLATE ANALYSIS

The technique used was a modification of the technique described by Shah and Wright (1974). Their technique involved the adsorption of glycolate from water samples on to activated alumina, desorption of the glycolate into a smaller volume of H_2SO_4, filtration of the H_2SO_4 to remove alumina and spectrophotometric determination of glycolate in the H_2SO_4 extract by reaction with a specific colorimetric reagent, 2,7-dihydroxynaphthalene (DHN). Calkins (1943) reported that neither formic, acetic, oxalic, succinic, tartaric, citric, benzoic nor salicylic acid reacted with DHN to produce a colour change. The efficiency of absorption and desorption of glycolate were monitored by addition of a range of known concentrations of glycolate to water samples in quadruplicate.

The following is a discussion of the technique which was used here with particular reference made to modifications of Shah's and Wright's technique.

(a) Glycolate Absorption

In the field, as soon as possible after sample collection, 1,000ml of lake water was filtered, under vacuum from a hand pump, through glass fibre filter paper (Whatman GF/C, nominal pore size 1.0μm) held in a 47mm diameter glass filter funnel (Millipore). Vacuum was kept to less than 13 kPa to minimise damage to algal cells (see section 2.4, above). Several changes of filter were necessary. The filters were used for chlorophyll extraction and the filtrate was divided equally between four 250ml polyethylene wash bottles. To each wash bottle sodium glycolate, from a stock of sterile sodium glycolate solution, was added to give the following range of final concentrations: 0, 6.4, 12.8 and 19.2 μgCl^{-1}. The wash bottles were shaken and the contents of each passed, by siphon action, through a glass column containing 6g of activated alumina (BDH, Brockman Grade II), which had been previously washed in 10% H_2SO_4, rinsed thoroughly in distilled water and dried, as described in Shah and Wright. The column arrangement is shown in figure 2.6/1. At the commencement of flow the columns were shaken thoroughly to evenly wet the alumina. Through flow time was approximately three quarters of an hour. After the

water had passed through the column, excess water was removed by suction from the bottom of the column with a 50ml syringe. Alumina was pushed from the column and stored in a sealed container, either in the dark at 0-4°C with 1ml methanol as a preservative, or frozen.

Shah and Wright did not use columns. Rather, they added alumina to flasks of filtered lake water and shook for 20 minutes. Use of columns was made necessary by the absence of a shaker for field work. As the technique is internally standardised, the effect of the column on absorption efficiency is irrelevant. Shah and Wright also processed alumina immediately rather than storing it. However, Fogg, Burton and Coughlan (1975) using the same technique as Shah and Wright to measure glycolate concentrations in some Antarctic lakes, stored alumina frozen for up to three months with no apparent loss of glycolate. The use of methanol as a preservative was necessary for samples from Lake Fryxell because of the unreliable nature of freezing facilities in the Lake Fryxell area. Methanol had no apparent effect on samples over 0-4 days, but beyond this time its effect was not tested. Lake Fryxell samples were stored for approximately two months before further processing and yielded an uncharacteristically low increase in colour development with increasing added glycolate concentration (see figure 4.0/12). The reasons for this are unclear but may be related to the use of methanol as a preservative with consequent loss of glycolate from the stored alumina. However, it is equally likely that this apparent loss of added glycolate was due to decomposition of glycolate upon storage of the sealed standards. These standards were made up to 100 $\mu g ml^{-1}$ in New Zealand and sterilised by autoclaving in sealed bijous. Autoclaving had no immediate effect on glycolate concentration. This was determined by assaying autoclaved and unautoclaved standard glycolate solution with 2,7-dihydroxy-naphthalene (Sigma). The standards used for samples from Lake Rotomanuka were frozen until needed whilst those for samples from Lake Fryxell were stored in the dark at 0-4°C for up to two months. Without knowing at which stage the apparent glycolate loss occurred in the Lake Fryxell samples, it is impossible to use the internal standardisation to calibrate the technique. This is discussed further in section 4. .

(b) Glycolate Extraction from Alumina

Each 6g lot of alumina was washed with two 100ml aliquots of distilled water and sucked dry on a GF/C filter in a Büchner funnel over full vacuum. The alumina was transferred to 30ml screw top polycarbonate centrifuge tubes (Sorval), 5ml of 12M H_2SO_4 (Univar, AR grade, Ajax Chemicals Ltd, Sydney) was added and the centifuge tube top was screwed down over a rubber O-ring. Centrifuge tubes were shaken at high speed on a flask shaker for one hour then centrifuged for four minutes at 6,000g in a Sorval RC2B centrifuge. The supernatant in each tube was removed and placed in a smaller centrifuge tube for further centrifugation at 6,000g for ten minutes to remove residual alumina particles.

Centrifugation seemed to be a vast improvement over the filtration technique which Shah and Wright used for cleaning their acid extracts. Centrifugation required fewer and less complex manipulations than filtration, which was initially tried. 12M H_2SO_4 was used instead of the 9M H_2SO_4 which Shah and Wright used, because 12M H_2SO_4 was found, during initial experimentation, to give better glycolate extraction.

Extraction presented the major problem encountered with this technique. High speed shaking was required to mix the viscous alumina/H_2SO_4 mixture. If this mixture was not shaken hard enough or was allowed to stand too long (more than approximately one hour) before centrifugation, it set rock hard. The results from Rotomanuka for 15.10.80 and 12.11.80 were ruined by insufficient shaking resulting in setting.

(c) Colorimetric Assay

Triplicate 0.15ml aliquots of each glycolate extract were added to 1.0ml of 0.01% DHN in concentrated H_2SO_4, in 10 x 100mm pyrex test tubes. Reagent blanks consisted of 0.15ml of 12M H_2SO_4 in 0.01% DHN and extract blanks, to correct for the yellow coloration commonly absorbed from lake water by alumina, consisted of 0.15ml of extract in 1.0ml of concentrated H_2SO_4. Each blank was also prepared in triplicate. Sample and blank tubes were mixed on a vortex mixer

(Vortex Genie, Scientific Industries Inc., Springfield, Mass., U.S.A.) for 15 seconds each. Because of the density of H_2SO_4 prolonged and vigorous mixing was necessary to produce reproducible results. Tubes were then capped loosely with plastic test tube caps and heated in a boiling water bath for one hour. After removal they were cooled to room temperature in a cool water bath and 1.0ml of 1.0M H_2SO_4 was added to each tube. After thorough mixing on a vortex mixer the contents of each tube were transferred to a cuvette for O.D. measurement at 490nm in a Beckman model 24 double beam spectrophotometer with 50% H_2SO_4 in the reference cell.

(d) Calculation of Glycolate Concentration

The mean $O.D._{490}$ values of each of the two types of blanks were calculated and summed. This total blank was subtracted from the mean $O.D._{490}$ value of the sample tubes, which contained the extract and DHN. When this corrected O.D. 490 was plotted against the concentration of glycolate added to the 250ml aliquot of lake water in the field, a linear relationship resulted (see figure 3.2/17). The intersection of this straight line with the x-axis was at a glycolate concentration corresponding to that existing in the lake water prior to the known additions.

Calculation of the error of estimation was by means of equation 4 in section 2.2.8 which gives the 95% confidence limits of the x intercept.

2.7 INT REDUCTION/EPIFLUORESCENT MICROSCOPY

In 1978 Zimmerman, Iturriaga and Becker developed a technique for distinguishing between metabolically active and inactive bacteria during microscopic observation. This technique was based on the fact that the formazan produced by reduction of INT (2-(*p*-iodophenyl)3(*p*-nitrophenyl) -5 phenyl tetrazolium chloride) by biological electron transport systems, is insoluble and forms a coloured region in or attached to those cells which are capable of reducing INT. Microscopic examination of cells for the presence or absence of black formazan dots enables cells to be classified according to the presence or absence, within a cell, of an active electron transport system.

In view of the above, it seemed that such a technique would be ideal for a subdivision of bacterial cells into those which could be responsible for substrate uptake (active metabolism) and those which would be very unlikely to be responsible for substrate uptake (inactive metabolism). To investigate the possibility of using this technique some preliminary experiments were performed on exponential phase pure cultures of common laboratory organisms. Zimmerman *et al* performed their investigation on natural water samples.

The method of Zimmerman *et al* involved incubating water samples at a final INT concentration of 0.2% for 20 minutes, fixing with a final concentration of 1% formaldehyde, filtering on to 0.1µm pore size Nuclepore polycarbonate membrane filters, staining with acridine orange and counting total cells by epifluorescence microscopy, and black dots in the same field by transmitted visible light (bright field) microscopy. By alternating the lighting systems the association of black dots with cells could be verified. 0.1µm filters were used because they are thinner (5µm c.f. 10µm) than 0.2µm filters. This, and their smaller pore size provide less background filter structure to interfere with transmitted light microscopy. However, 0.1µm filters have slower filtration rates than 0.2µm filters. It was found when using the staining technique described in section 2.3 that sample staining and filtration took up to one hour per sample.

A comparison of unfiltered INT-treated pure cultures using a combination of phase contrast and bright field microscopy soon revealed a major limitation of the technique of Zimmerman *et al*. Some cells,

including *Escherichia coli* and *Staphylococcus aureus* produced a number of small regions of formazan per cell rather than just one large one, as was produced by *Pseudomonas fluorescens*. These small regions were plainly visible during phase contrast or bright field examination of wet mounts but were very difficult to see against a filter background using bright field microscopy. This resulted in a decrease in the percentage of apparently active cells of between 50 and 75%. Because of this limitation, it was decided that the results obtainable using the technique of Zimmerman *et al* would not be valuable enough to justify the extra time involved in obtaining them.

2.8 TEMPERATURE, OXYGEN AND LIGHT MEASUREMENT

In Lake Rotomanuka both temperature and oxygen were measured with a Y.S.I.54 combined oxygen and temperature meter (Yellow Springs Industries Co., Yellow Springs, Ohio, U.S.A.). This meter was calibrated and operated according to the manufacturer's instructions.

At Lake Fryxell oxygen was measured with a Delta Scientific Model 2010 Automatic Dissolved Oxygen Analyser (Delta Scientific Corp., Lindenhurst, N.Y., U.S.A.). This meter was calibrated in the air to approximately half-sensitivity, in order that readings would be on the scale when taken in the oxygen-supersaturated photic zone of Lake Fryxell. Oxygen readings on the decalibrated meter were then multiplied by a factor equal to the extent of decalibration, e.g. at 1°C near sea level with the probe membrane wet, the meter was set at 5.8 ppm instead of 14.1 ppm as specified in the manufacturer's calibration tables. Readings were then multiplied by 14.1/5.8. The validity of this decalibration technique could only be checked at depths where oxygen concentration was less than 20 ppm (the upper range of the meter). At these depths, oxygen concentrations measured using either a properly calibrated meter or a decalibrated meter were within 1.0 ppm of each other. The relationship between oxygen measurements and true oxygen concentrations at oxygen concentrations which were greater than 20 ppm is unknown. In view of this it must be emphasised that the oxygen profile depicted in figure 4.2/2 is only semiquantitative.

Below 8.6m in Lake Fryxell, the smell of H_2S in water samples became apparent. Because of the detrimental effect of H_2S on oxygen meter electrodes, these were cleaned and a new membrane and electrolyte were used on each date on which the meter was used.

Temperature in Lake Fryxell was measured with a matched thermistor and meter.

Irradiance readings were made with a Li Cor Quantum/Radiometer/Photometer Model LI-185A (Li Cor Inc., Lincoln, Nebraska, U.S.A.) which measured photosynthetically active radiation.

Chapter 3

LAKE ROTOMANUKA

RESULTS AND DISCUSSION

3.1 INTRODUCTION

In order to examine the extent to which aquatic microheterotrophs modify their ability to take up substrates associated with algal extracellular release on a diurnal basis, studies of the heterotrophic uptake kinetics of glucose and glycolate were made in two lakes, Lake Rotomanuka and Lake Fryxell. Owing to the different nature of these two lakes, the different facilities available at each and to experience acquired during the course of these studies, a different methodological approach was adopted at each lake. This chapter deals with the work which was done on Lake Rotomanuka and the results obtained. The work on Lake Fryxell is discussed in chapter 4.

Lake Rotomanuka was chosen as a study site for two reasons:

(i) It is convenient to the University of Waikato and has vehicle access to the shore. During the course of my studies a shed and boat were available at the lake side for research purposes.

(ii) Lake Rotomanuka is the subject of algal population and biomass studies by the N.Z. Ministry of Works and Development (Pridmore, *pers. comm.*) and studies of its physical and chemical parameters and its fish population are being carried out by the Biological Sciences Department of the University of Waikato (Boubée, 1978). Because of the on-going nature of these studies a body of experience of conditions in Lake Rotomanuka was accessible throughout the course of my research.

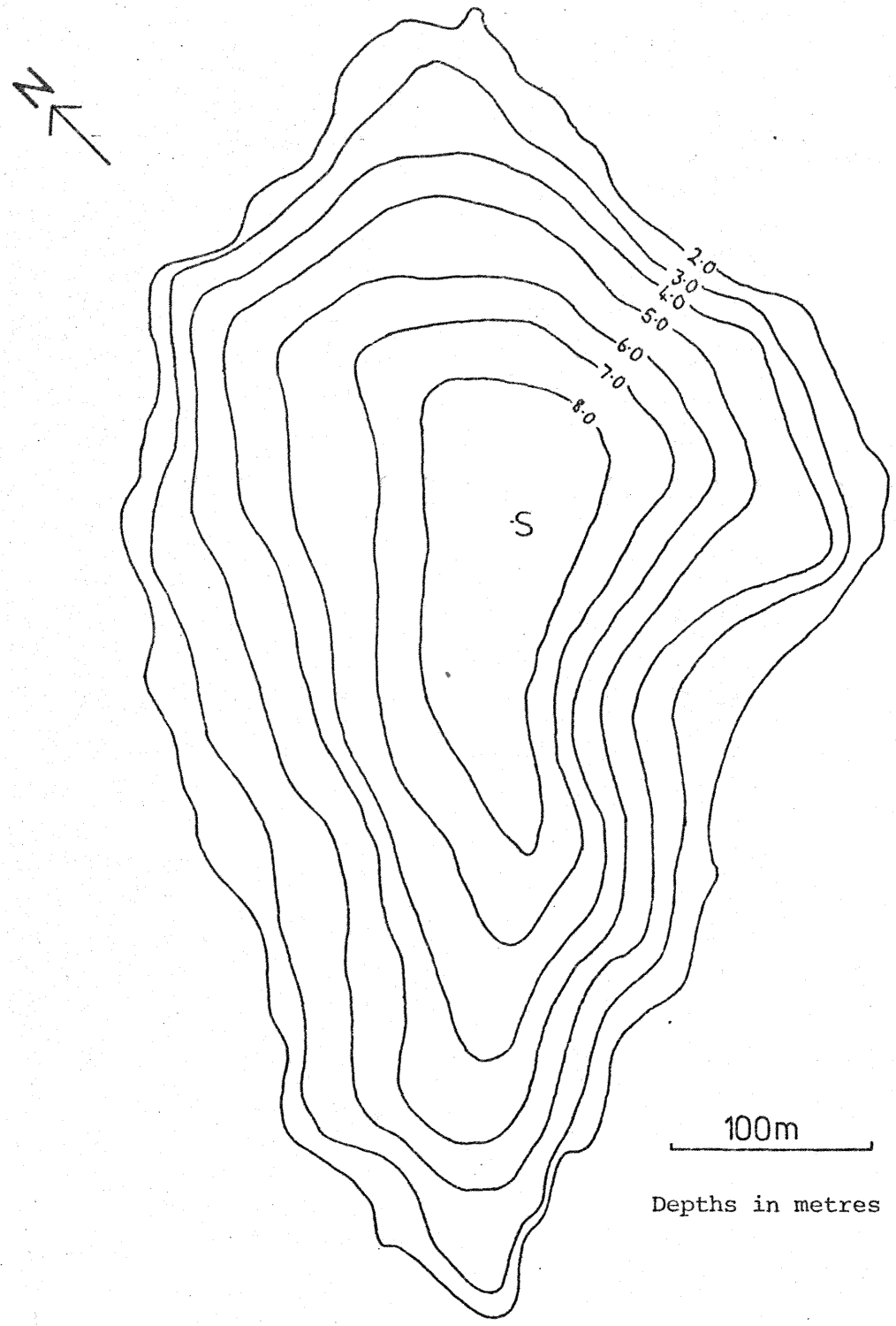


Figure 3.2/1 - Bathymetry of Lake Rotomanuka, showing sampling site.

(from Irwin, 1980)

3.2 DESCRIPTION OF LAKE AND SAMPLING SITE, EXPERIMENTAL APPROACH AND RESULTS

3.2.1 Lake Rotomanuka - Description

Lake Rotomanuka (37°57'S, 175°19'E) is a small, warm monomictic lake of 13.7 ha surface area. It is situated in the Waikato Basin, New Zealand, approximately one kilometre south of the township of Ohaupo at an altitude of 50m. The lake has a mean depth of 5.02m (Green, *pers. comm.*) and a maximum depth of 9m and is bounded on three quarters of its perimeter by pastoral farmland. The remaining perimeter abuts an area of raupo (*Typha* sp.) swampland.

The state of stratification at each sampling time is shown in figures 3.2/2 and 3.2/3.

3.2.2 Sampling Site and Sampling Procedure

All samples were taken from the same sampling point at the deepest part of the lake (see figure 3.2/1). To facilitate finding this point in the dark it was marked by a fixed buoy over each 24 hour sampling period. The buoy was positioned by lining up landmarks.

Bulk water samples were taken with a 10 litre plankton sampler which was emptied through a 100µm plankton net into a 2.5 litre glass bottle from which subsamples were taken for subsequent incubation. The sampler and 2.5 litre bottle were rinsed thoroughly in lake water between samples.

3.2.3 Experimental Approach

Initially it was intended that the heterotrophic uptake kinetics of both glucose and glycolate be measured at each of four times of the day on a succession of dates at regular intervals during the summer of 1980/81. At each sampling time the naturally occurring concentration of glycolate was to be measured. It was assumed, perhaps unwisely, that the surface few metres of the lake were always well enough mixed to ensure that the same heterotrophic population was studied at each time of day for each sampling date. Algal migration was checked for, unsuccessfully, using chlorophyll extractions and the occurrence of changes in bacterial numbers which were not diurnally cyclic, was checked for by using bacterial direct counts.

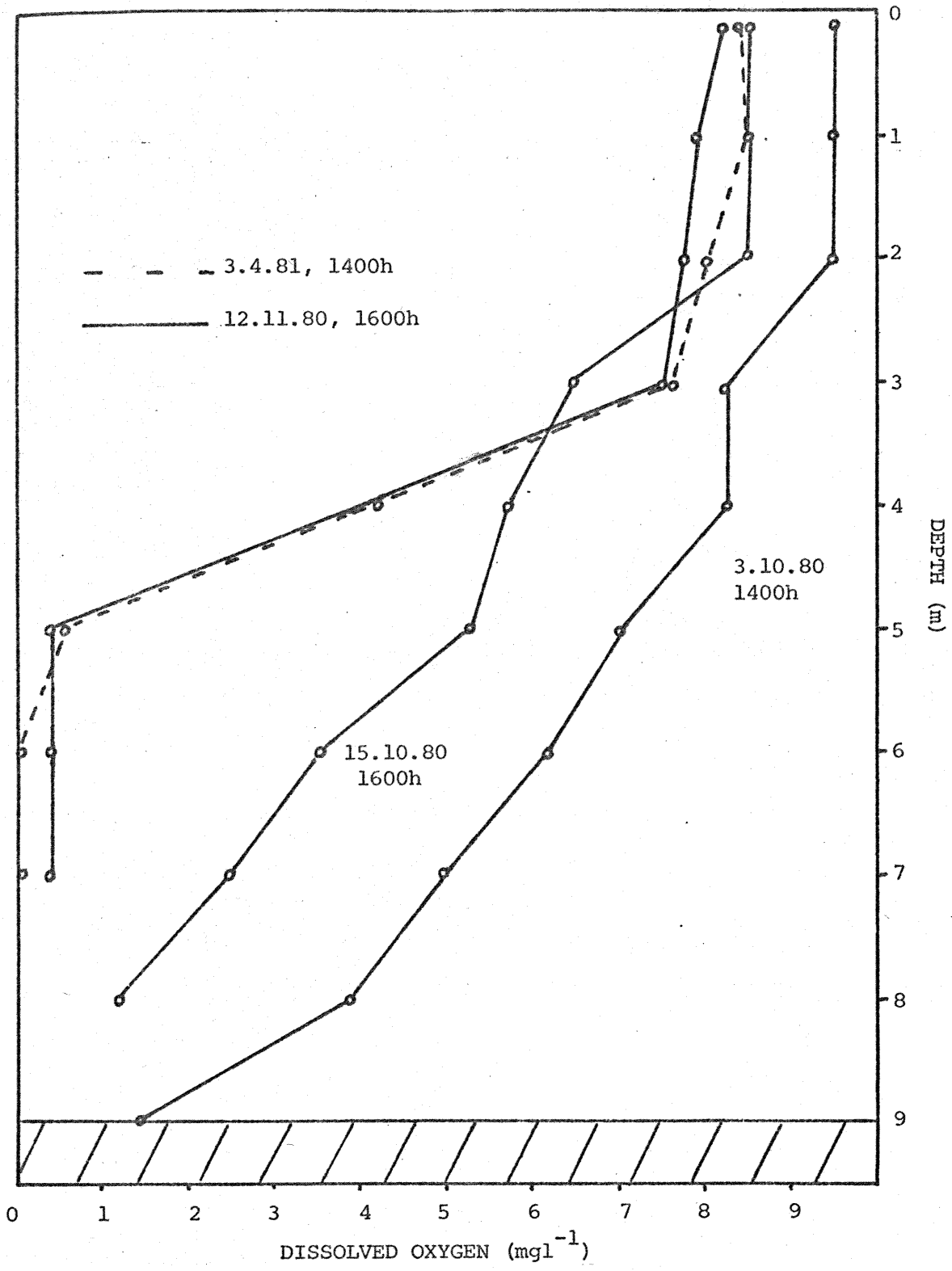


Figure 3.2/3 - Dissolved Oxygen Profile
Lake Rotomanuka

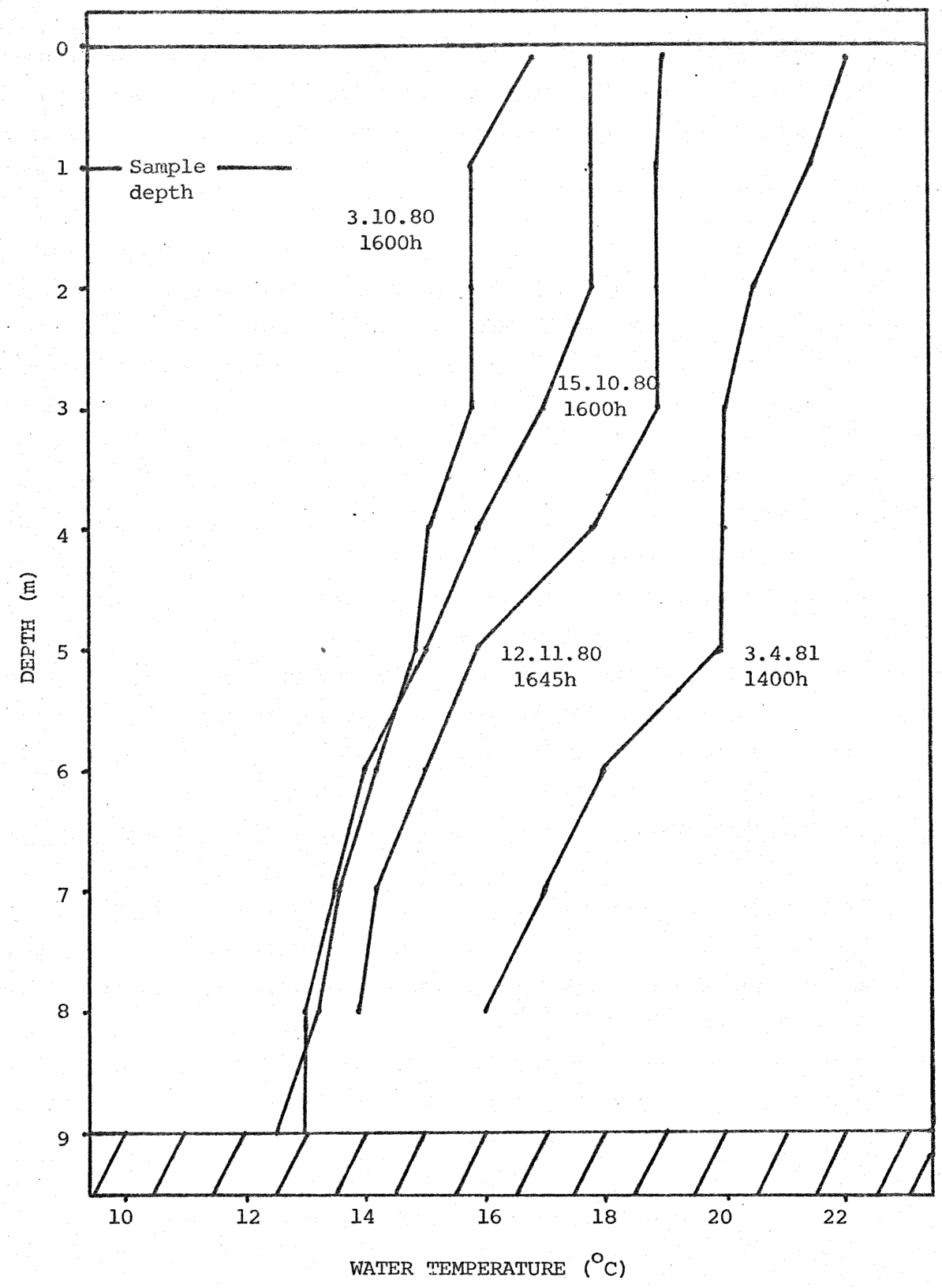


Figure 3.2/2 - Water Temperature Profile

Lake Rotomanuka

Because of the large number of replicate incubations which were found to be needed to produce statistically significant comparisons between different results, samples were able to be taken at fewer times on fewer dates than anticipated.

3.2.4 Results and Discussion

In the following results only total uptake of substrate is given. In some cases this is based on the measurement of respired $^{14}CO_2$ from all the samples in a particular study with a factor for the fraction of activity incorporated into biomass being calculated from a lesser number of filtrations. At no time, when both incorporated ^{14}C and respired $^{14}CO_2$ were measured over a range of concentrations or times, was there any apparent variation in the partitioning of activity with respect to changing time or concentration. This is true for the results from the first, third and fifteenth of October, 1980 (see figures 3.2/4, 5, 6, 7 and 8) and from the first, third and fourth of April 1981 (see figures 3.2/14, 15, 16, 18 and 19). At this stage it would be pertinent to point out that where dates have been abbreviated, it is in the form of day/month/year, not month/day/year.

3.2.4.1 Uptake of Glucose and Glycolate on 1 October 1980 (1/10/80) and 3 October 1980 (3/10/80)

On 1/10/80 it was established that the uptake rates of both glucose and glycolate did not vary with time over the period of time for which measurements were made (see figure 3.2/4). From these results suitable incubation times for heterotrophic uptake studies were calculated. On 3/10/80 the characteristics of the heterotrophic uptake of glucose and glycolate were measured at four different times of day. Each measurement involved only four concentrations of added substrate and no replicates. The results are expressed as graphs of uptake versus concentration and as modified Lineweaver-Burke plots in figures 3.2/5 and 3.2/6. The kinetic parameters of V_{max} (maximum attainable uptake rate), T_t (turnover time) and $(K_T + S_n)$ (the sum of the uptake rate constant and the concentration of naturally occurring substrate), calculated from these curves are shown in figures 3.2/20 and 3.2/21 where they are plotted against the time of day. The error bars represent the upper and lower 95% confidence limits for each point and are calculated as described in section 2.2.8.

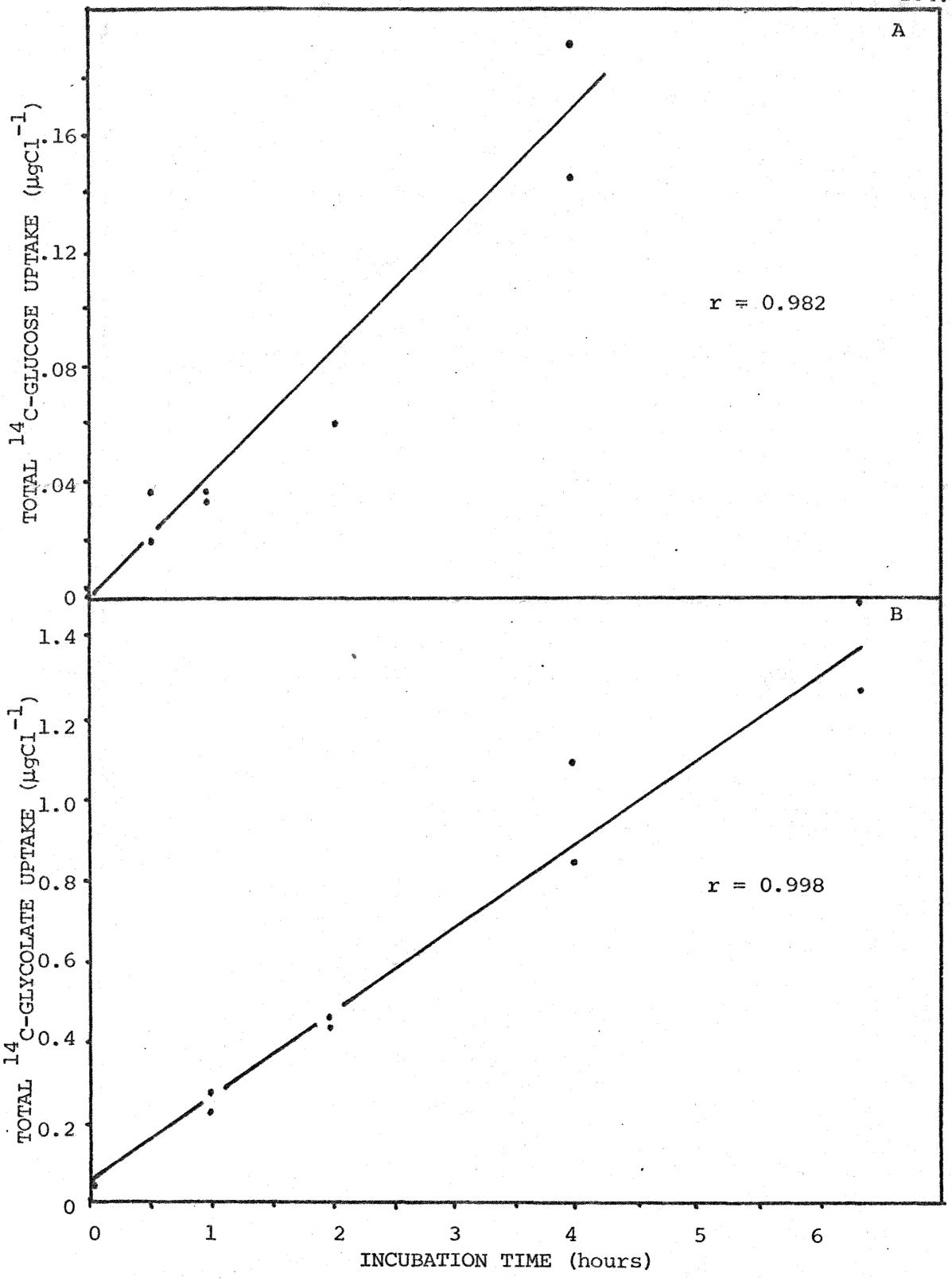


Figure 3.2/4 - The Effect of Incubation Time on Substrate Uptake

Lake Rotomanuka 1045h 1.10.80

A - Glucose uptake at 2.0 μgCl^{-1} added glucose

B - Glycolate uptake at 108 μgCl^{-1} added glycolate

Incubation Temperature 17.5°C

Zero time was formalinised 90 minutes prior to substrate addition

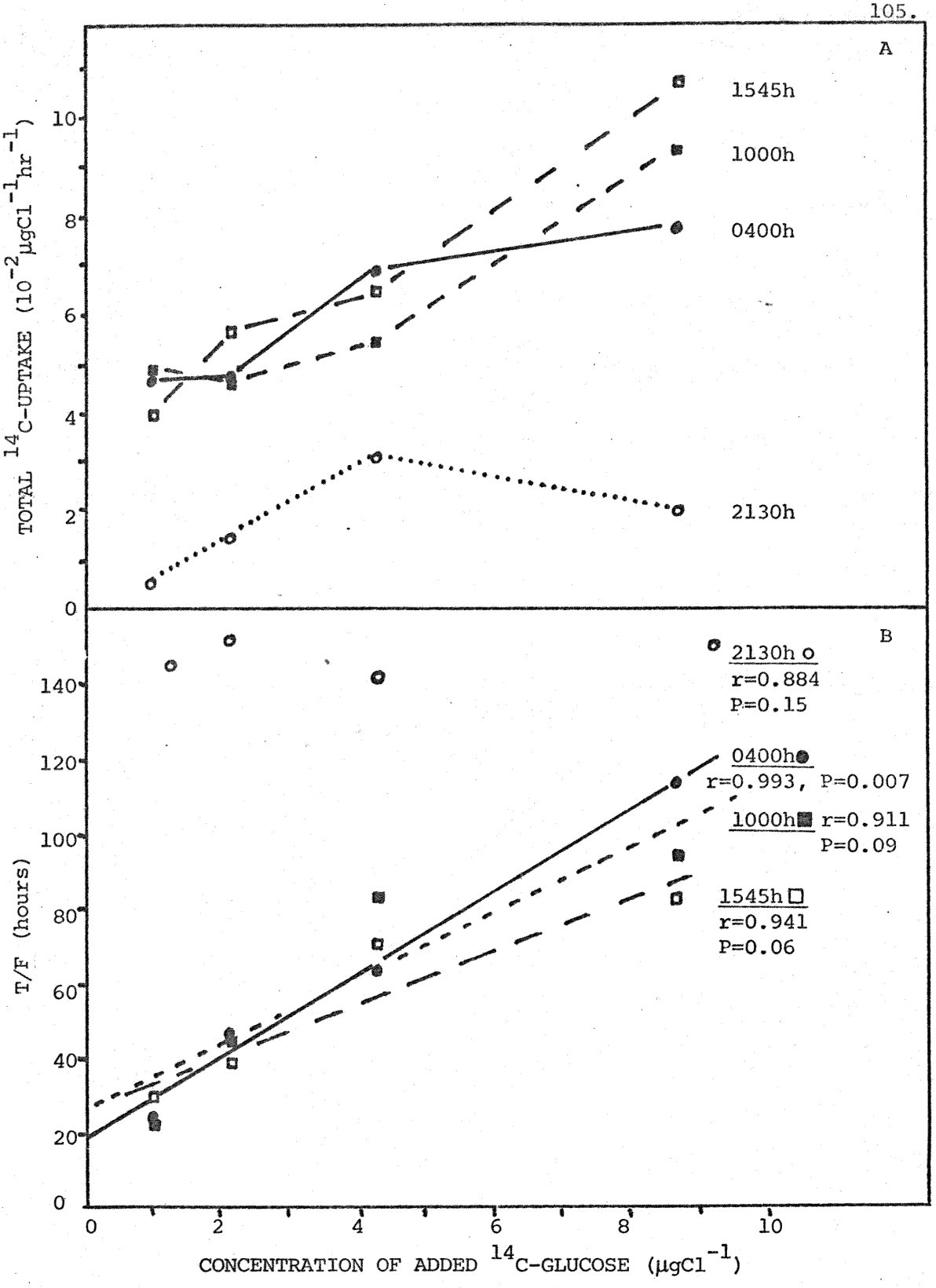


Figure 3.2/5 - Uptake of ^{14}C-Glucose in Lake Rotomanuka
0400h, 1000h, 1545h, 2130h 3.10.80

A - Total Uptake, Respired plus incorporated ^{14}C-activity

B - Incubation time/Fraction of added ^{14}C-activity which was taken up

Incubation Temperatures 0400h - 15.5°C, 1000h - 16.0°C
1545h - 16.5°C, 2130h - 15.5°C

Incubation Times All 2hrs

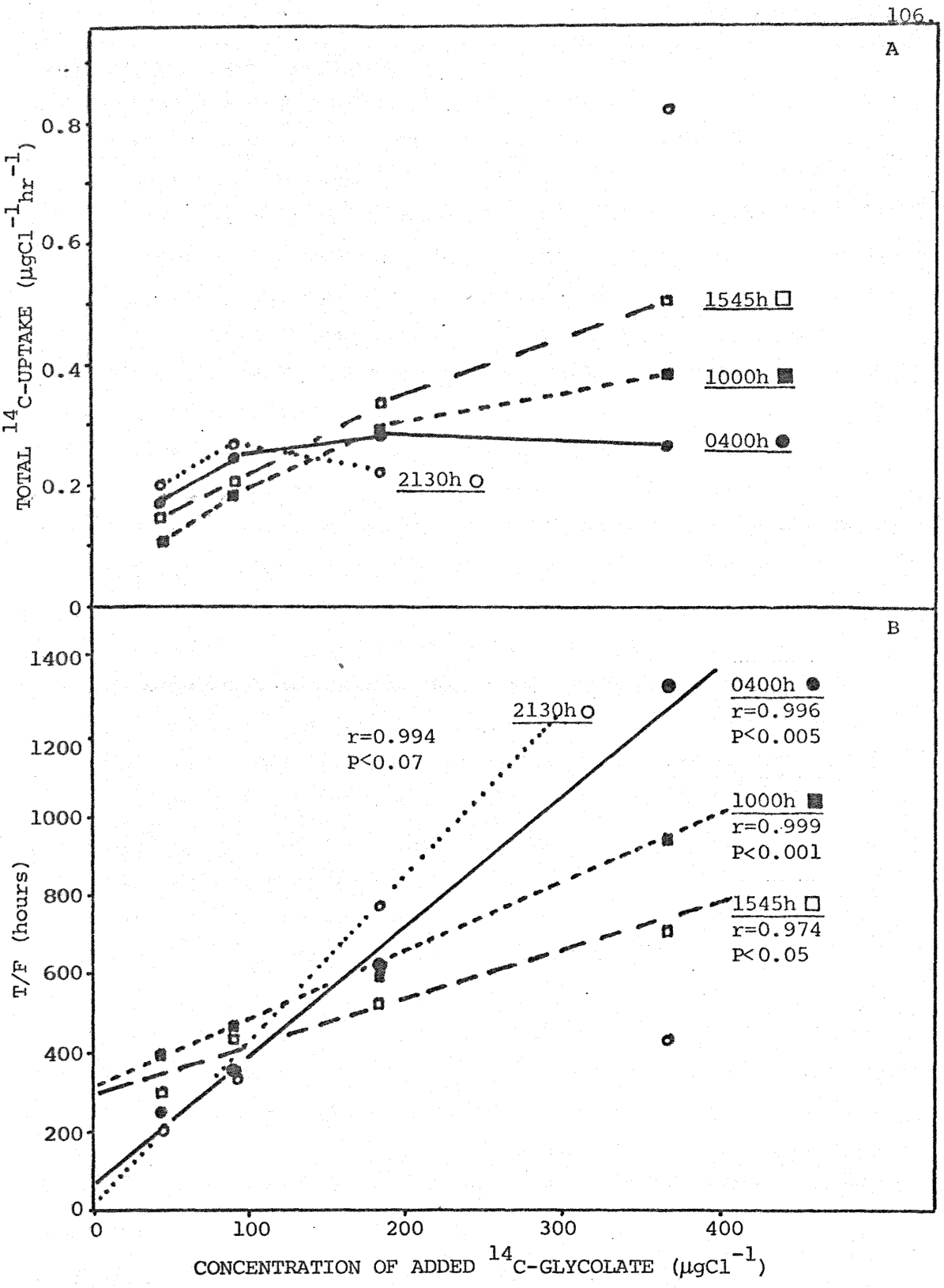


Figure 3.2/6 - Uptake of ^{14}C-Glycolate in Lake Rotomanuka

0400h, 1000h, 1545h and 2130h 3.10.80

A - Total Uptake. Respired plus incorporated ^{14}C-activity

B - Incubation time/Fraction of added ^{14}C-activity which was taken up

Incubation Temperatures 0400h - 15.5°C, 1000h - 16.0°C
1545h - 16.5°C, 2130h - 15.5°C

Incubation Times All 3hr 45min

Heterotrophic Uptake of Glycolate

Two main features emerged from this experiment:

(a) The uptake kinetics appeared to change over a time period of less than 24 hours.

(b) There was a need for more data points in each curve. In this experiment it was impossible to distinguish between a possible cyclic trend with V_{max} being higher during the night and lower during the day, and a longer term trend where V_{max} increases continuously throughout the 24 hours of the experiment. This problem arose because it was not possible to fit a straight line to all four of the data points obtained at 2130h. By omitting either the third or the fourth point from consideration either one of the trends above could be obtained. The fraction of activity respired at each of these two concentrations was the same, suggesting that experimental error was not the source of the apparent anomaly. It was possible that neither point was anomalous and that there were two trends responsible for the change in kinetics throughout the day, one exerting more effect at low concentrations of substrate and the other at high concentrations.

Heterotrophic Uptake of Glucose

As occurred in the case of glycolate, there was change in the uptake kinetics for glucose over 24 hours (see figure 3.2/5).

At only one time, 0400h, were the results interpretable in terms of Michaelis-Menten kinetics. At the other times no such kinetic interpretation could be made using the modified Lineweaver-Burke relationship. The lines fitted to these points were not significant at the 5% level. This was reflected in the very large 95% confidence limits for the kinetic parameters which were calculated from these lines (see figure 3.2/21).

Subjectively, the change in kinetics appeared to be due mostly to an increase in uptake at higher concentrations during daylight hours. This could be explained in terms of increased activity of a low affinity, possibly algal, uptake system during the day. Wright and Hobbie (1966) demonstrated the existence of such a system which, when present in lake water samples, was responsible for a deviation from Michaelis-Menten kinetics in which the uptake rate became progressively

greater than the calculated rate as the substrate concentration was increased. Wright and Hobbie (1966), Wright (1971) and Krambeck (1979) all suggested that the uptake rates from the lowest possible added substrate concentrations should be used for the calculation of kinetic parameters because uptake kinetics at the lowest substrate concentrations are more representative of the uptake kinetics of the systems which take up most of the substrate at natural substrate concentrations. If, on these grounds, the uptake rate for glucose at its highest added concentration (9 μgCl^{-1}), is excluded from consideration, then there is no apparent nor significant difference between the modified Lineweaver-Burke plots obtained at 0100, 1000h and 1545h (see figure 3.2/5). However, the 95% confidence limits of the kinetic parameters which were calculated from these reduced data sets (see figure 3.2/21) are so wide that quite large changes in uptake kinetics might be masked.

The apparent trend of an increase in uptake rate, at 9 μgCl^{-1} of added glucose, during the day and a decrease at night is similar to the trend in the chlorophyll concentrations at the sampling depth. It is not known whether the change in chlorophyll concentrations reflected a change in algal population or in algal chlorophyll content (see section 2.4). Either a change in the algal population or in algal metabolism might account for the apparent trend in the uptake rate at 9 μgCl^{-1}.

I have no explanation for the drastic reduction in the uptake rates measured at 2130h. There is no way of knowing whether this reduction was caused by a heterogeneous distribution of heterotrophs in the water mass or by a change in the uptake kinetics of a constant heterotrophic population. No significant change in bacterial numbers occurred between 1545h and 2130h (see figure 3.2/22D).

3.2.4.2 Uptake of Glycolate on 15 October 1980

To enable more data points to be calculated for each curve, only glycolate uptake was studied and only at two different times over a 24 hour period. A wide range of concentrations was used so that the effect of the concentration range on the final result could be examined.

The results for the experiment done at 0430h are shown in figure 3.2/7; those for the experiment done at 1540h are shown in figure 3.2/8. Four points emerge from these results.

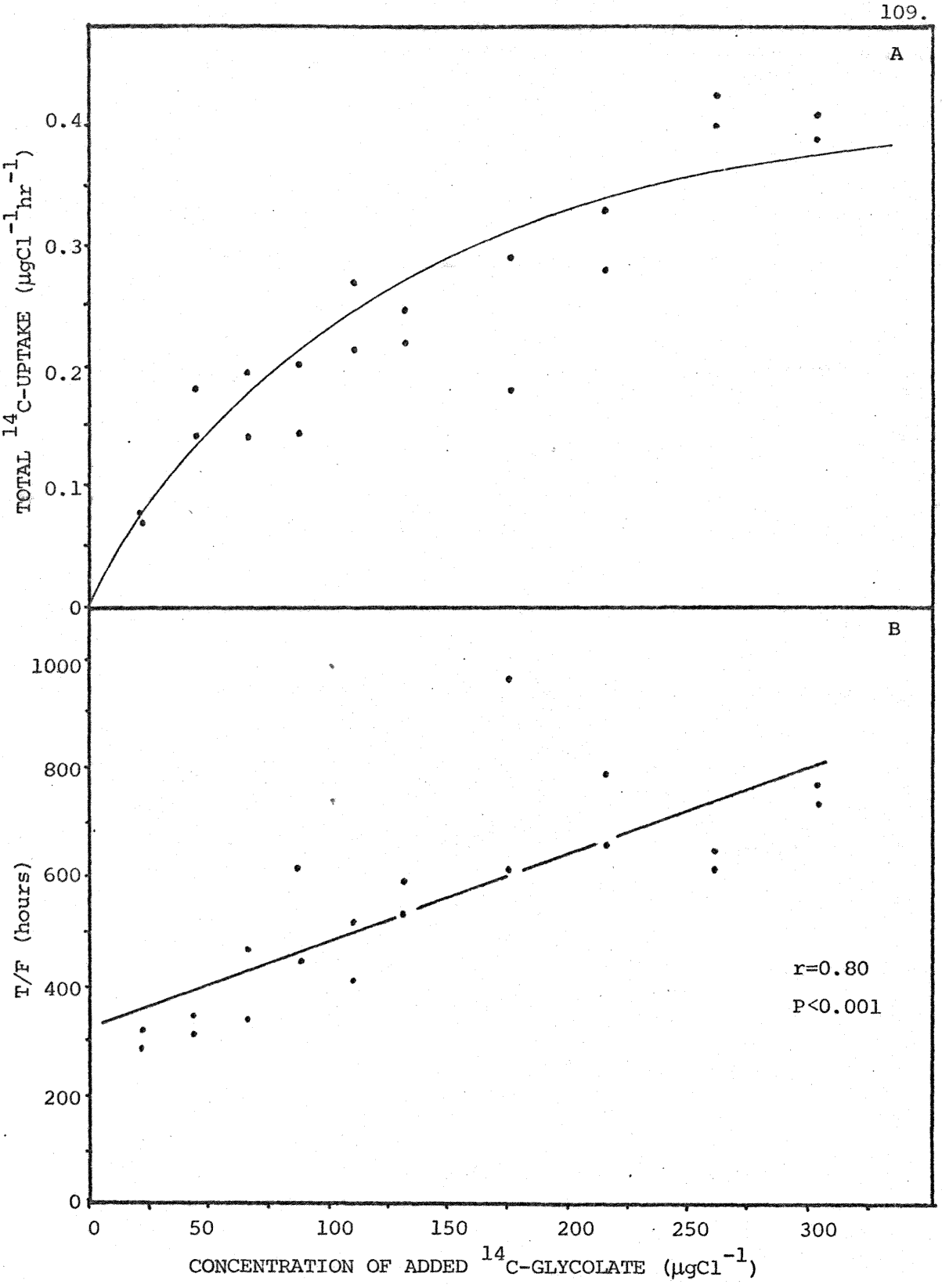


Figure 3.2/7 - Uptake of ^{14}C-Glycolate in Lake Rotomanuka

0430h 15.10.80

A - Total uptake. Respired plus incorporated ^{14}C-activity

B - Incubation time/Fraction of added ^{14}C-activity which was taken up

Incubation Temperature 18°C

Incubation Time 3hr 50min

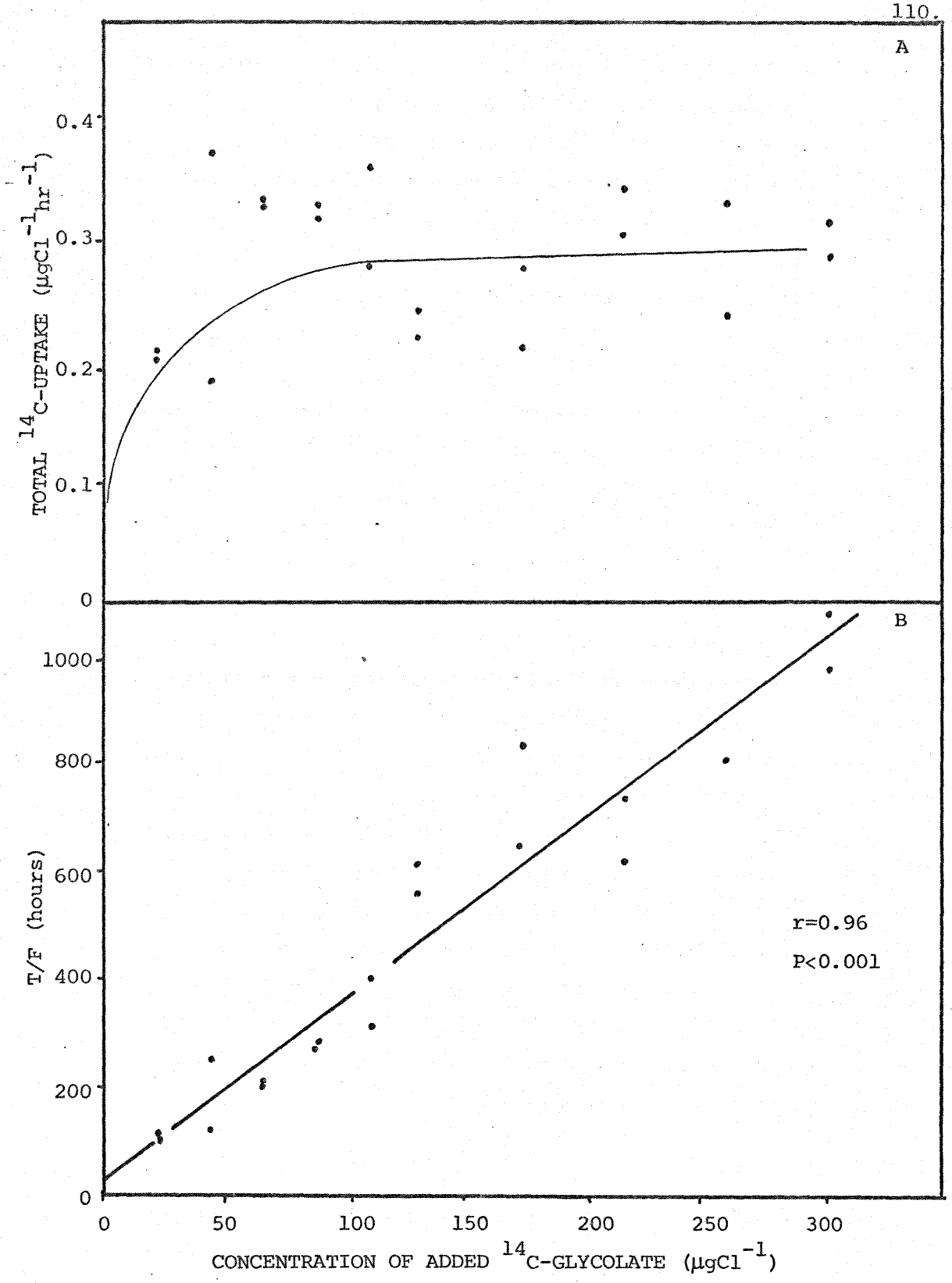


Figure 3.2/8 - Uptake of ^{14}C-Glycolate in Lake Rotomanuka
1540h 15.10.80

A - Total uptake. Respired plus incorporated ^{14}C-activity

B - Incubation time/Fraction of added ^{14}C-activity which was taken up

Incubation Temperature 17.5°C

Incubation Time 3hr 40min

(a) An obvious difference existed between the uptake kinetics at the two different times. Uptake at 0430h was characterised by a turnover time which was larger than the turnover time at 1540h. This difference was apparent and significant. There was no apparent difference between the V_{max} values at each time and although there was an apparent difference in the $(K_T + S_n)$ values, this difference was not significant at the 5% probability level.

(b) The direction of change and the type of change in the kinetic parameters did not resemble those obtained on 3/10/80 in which the values of all three kinetic parameters were higher during daylight hours.

(c) At 0430h the modified Lineweaver-Burke plot was not truly linear. This type of deviation from linearity, in which the slope increases towards the origin has been discussed by Williams (1973) and is presumably due to the overall kinetics measured being the sum of the kinetics of many independent uptake systems. Despite this apparent difference, there was no significant difference, at the 5% probability level, between the slope and intercepts of a line drawn through points obtained using glycolate concentrations of 22.4, 44.8, 66.9, 89 and 111 μgCl^{-1} and those of a line drawn through the points obtained using glycolate concentrations of 132, 176, 219, 262 and 304 μgCl^{-1}. Nor did using the data from the lower five concentrations, at each time, alter the apparent difference between the uptake kinetics at 0430h and those at 1540h.

(d) The results from 1540h showed an apparent decrease in uptake rate between 132 and 176 μgCl^{-1} of added glycolate. This could be explained by one of the uptake systems contributing to the overall uptake kinetics being inhibited by excess added substrate. However, this apparent trend may not be real. Only one of the three points in question was outside the 95% confidence limits for the scatter around the regression line. These confidence limits were calculated at the appropriate concentrations using equation 2 in section 2.2.8.

3.2.4.3 Uptake of Glycolate on 12-13 October 1980 over a 24h Period

As there was no significant difference between the results obtained using either the upper or the lower five concentrations at each time on 15/10/80, it was decided that for the experiment done over the 24h period 12 October 1980 to 13 October 1980, only the lower five concentrations would be used and that the saving in resources would be used to obtain data at five times of the day instead of two. Because in previous experiments the percentage of substrate respired to CO_2 had been shown to the independent of added substrate concentration, in the experiments of 12-13/10/80 this value was calculated using filtrations (to measure incorporation) for the highest concentration only of substrate at each time of day.

The results, which are shown in figures 3.2/9, 10, 11, 12 and 13, are discussed below.

(a) If one accepts the acceptibility criteria of Gillespie and Spencer (1980) that P is less than 0.05, then only two of the sets of results, those for 1645h and 2230h on the 12th October, are explainable in terms of Michaelis-Menten kinetics. However, at 1645h on the 12th October a deviation from the Michaelis-Menten relationship is apparent in which the slope of the modified Lineweaver-Burke plot decreases towards the origin (figure 3.2/9). This type of deviation may have been due to a number of causes:

(i) Depletion of substrate at low concentrations of added substrate (Bell, 1980). However, as the amount of substrate incorporated and respired did not exceed 5% of the added substrate at any concentration this cause seems unlikely.

(ii) The finite time period required for the pool of metabolic precursors to the respiration pathway to attain isotopic equilibrium (Bell, 1980). As the concentration of labelled substrate decreases this equilibrium time becomes longer, with the result that a lower percentage of the label is found in respiration products and the ratio of the ^{14}C label found as $^{14}CO_2$ and the ^{14}C label found as biomass on the filter is no longer constant with changing substrate concentration. Unfortunately

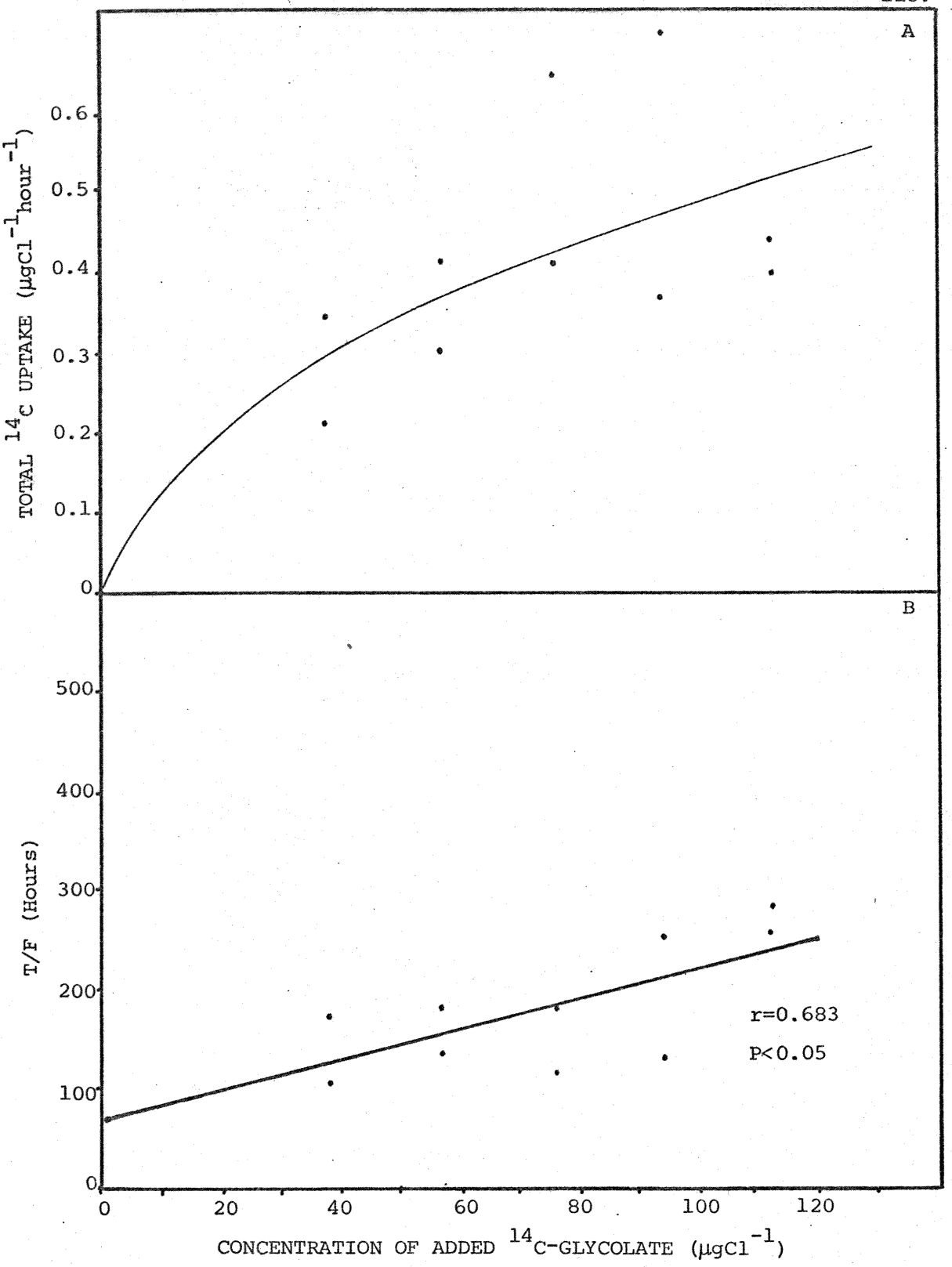


Figure 3.2/9 - Uptake of ^{14}C-Glycolate in Lake Rotomanuka

1645h 12.11.80

A - Total uptake. Respired plus incorporated ^{14}C-activity

B - Incubation time/Fraction of added ^{14}C which was taken up

Incubation Temperature 20°C

Incubation Time 2hr 40min

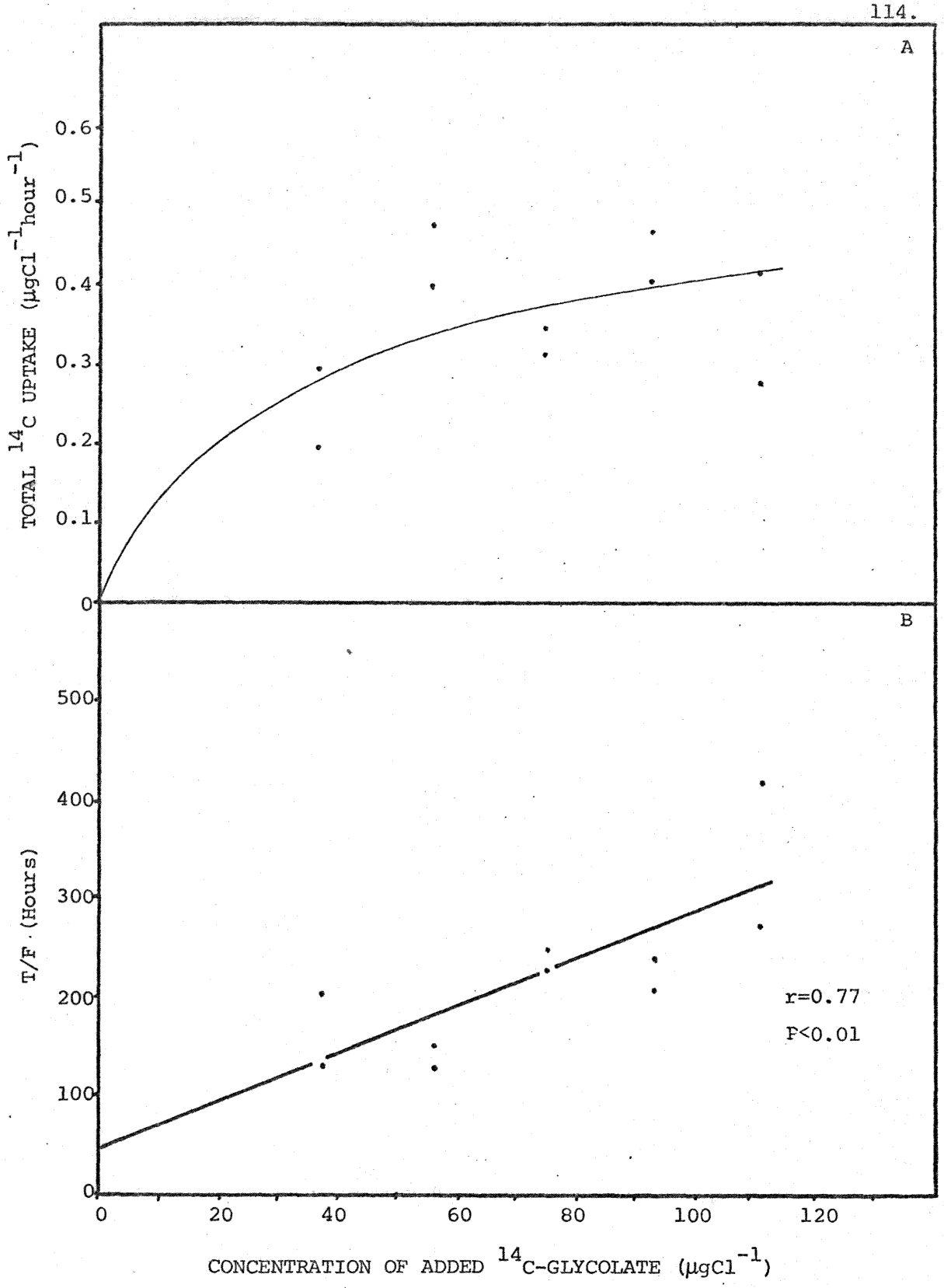


Figure 3.2/10 - Uptake of ^{14}C-Glycolate in Lake Rotomanuka

2230h 12.11.80

A - Total uptake. Respired plus incorporated ^{14}C-activity

B - Incubation time/Fraction of added ^{14}C which was taken up

Incubation Temperature 19.5°C

Incubation Time 2hr 50min

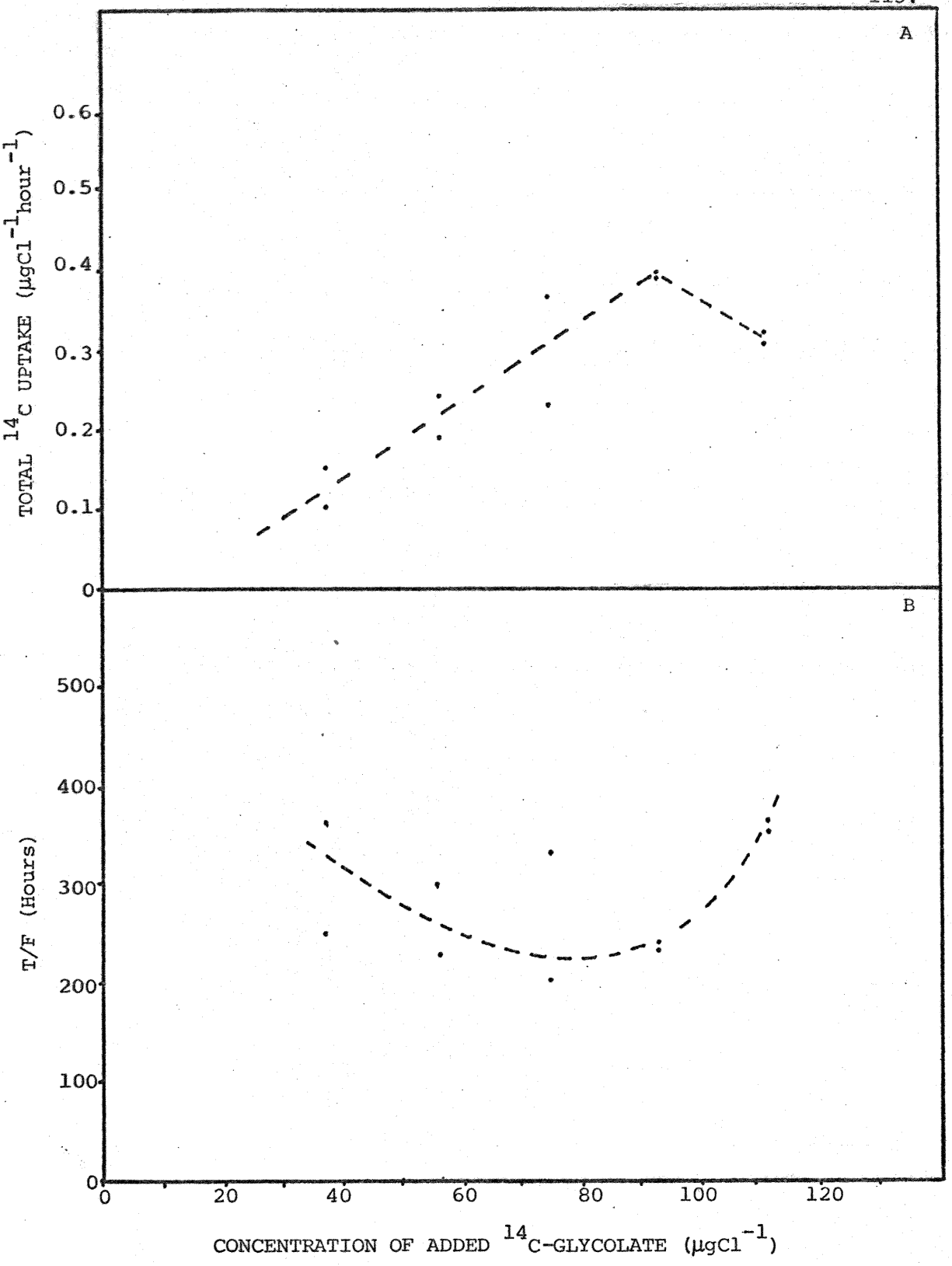


Figure 3.2/11- Uptake of ^{14}C-Glycolate in Lake Rotomanuka

0530h 13.11.80

A - Total uptake. Respired plus incorporated ^{14}C-activity

B - Incubation Time/Fraction of added ^{14}C which was taken up

Incubation Temperature 18°C

Incubation Time 2hr 50min

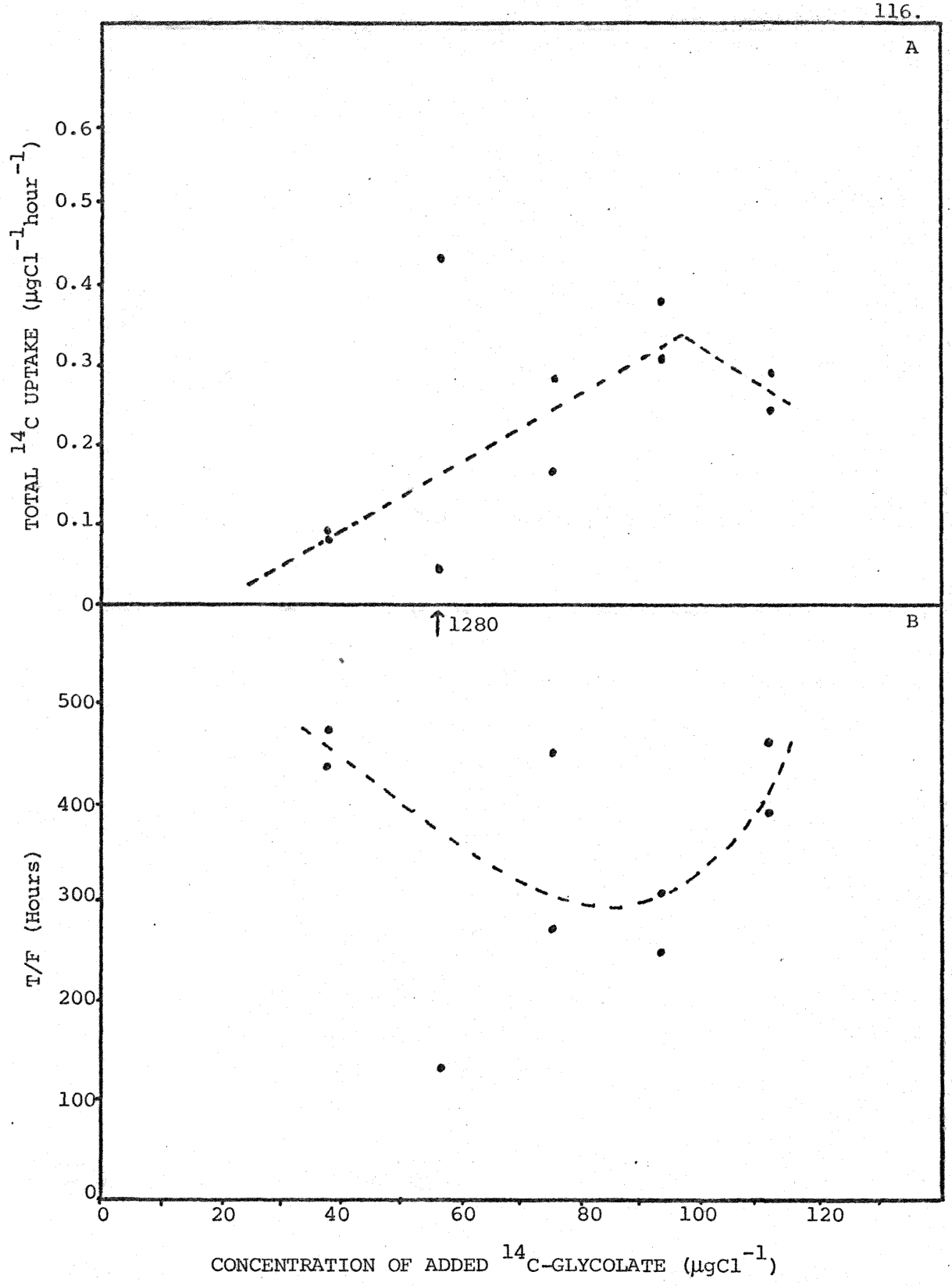


Figure 3.2/12- Uptake of ^{14}C-Glycolate in Lake Rotomanuka

1100h 13.11.80

A - Total uptake. Respired plus incorporated ^{14}C-activity

B - Incubation time/Fraction of added ^{14}C which was taken up

Incubation Temperature 18°C

Incubation Time 3hr 0min

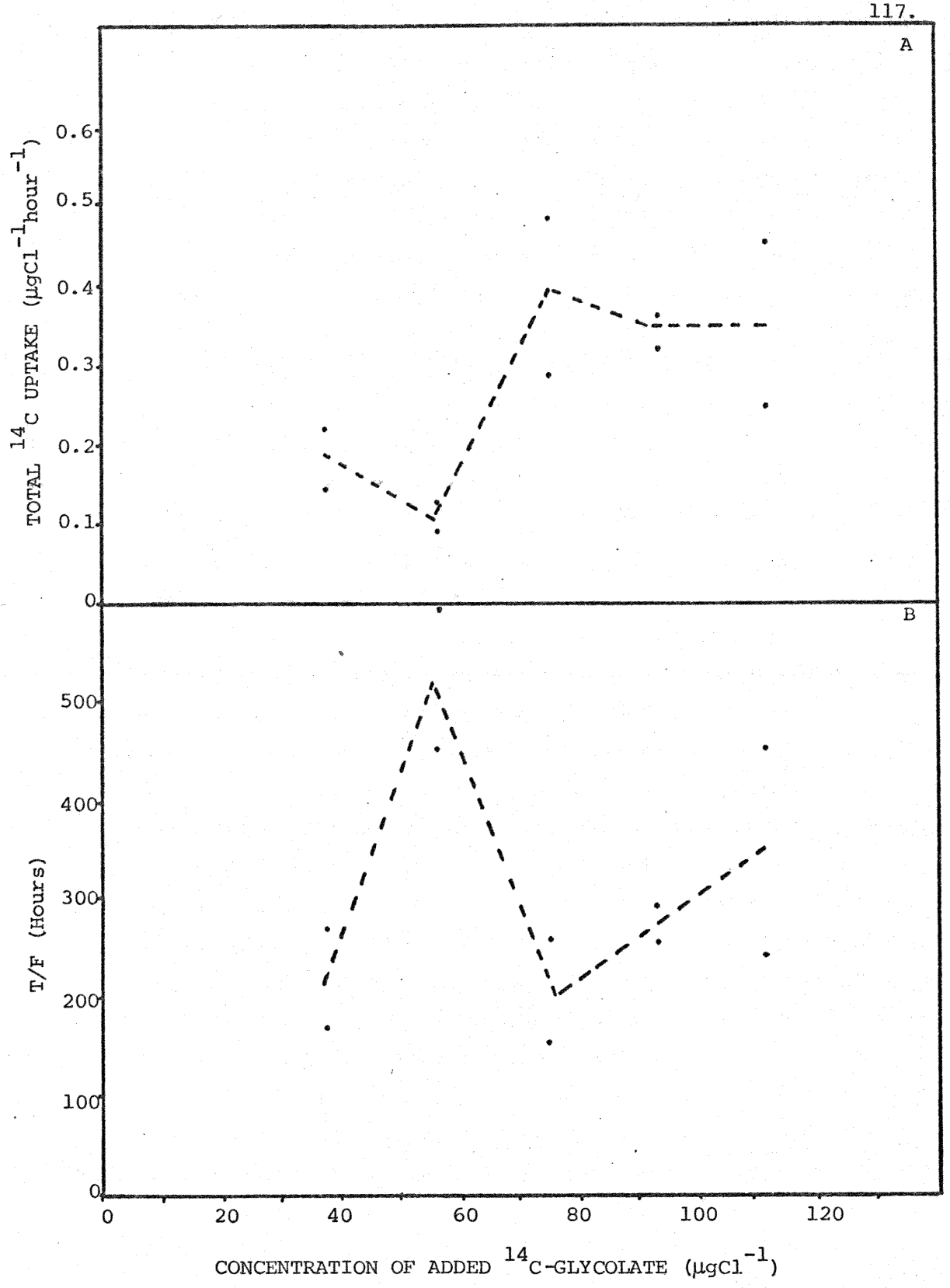


Figure 3.2/13- Uptake of ^{14}C-Glycolate in Lake Rotomanuka

1600h 13.11.80

A - Total uptake. Respired plus incorporated ^{14}C-activity

B - Incubation time/Fraction of added ^{14}C which was taken up

Incubation Temperature 18.5°C

Incubation Time 2hr 50min

this effect can be neither confirmed nor discounted in this case as the value for the fraction respired was obtained at only one concentration.

(iii) The increase in slope of the modified Lineweaver-Burke plot away from the origin may be due to an inhibitory effect on uptake of higher concentrations of added substrate. A similar anomalous decrease in uptake is seen at the same concentration of added glycolate at four out of the five times of day in the experiments described here.

(iv) Scatter of points about the line may also account for the deviation seen.

(b) At 0530h, 1100h and 1600h on the 13th of October, neither of the uptake curves bear any resemblance to typical Michaelis-Menten curves. Instead they suggest a sigmoidal relationship between uptake and concentration. A similar relationship was reported by Vaccaro and Jannasch (1967) for the uptake of glucose in the Pacific Ocean. This phenomenon is also discussed by Krambeck (1979) who explains it by saying that the metabolic pathways branching from the main pathways of utilisation of the substrate might be controlled by allosteric enzymes, i.e. the side reactions do not operate significantly until a threshold concentration of substrate has been exceeded. As substrate utilisation within the cell maintains the substrate concentration gradient across the cell membrane this threshold effect is seen in the cell's uptake kinetics for the substrate.

(c) Even without being able to quantify the kinetic parameters at most of the times of day, it is apparent that there are changes in uptake kinetics throughout the 24 hour period. However, the changes in kinetics are not based on a regular dirunal cycle, as the results for 1600h; 12/11/80 are quite different from those from the same time on the following afternoon.

3.2.4.4 Uptake of Glucose and Glycolate on 1 January 1982

The relationship between uptake and time was again confirmed as being linear (see figure 3.2/14). Note that for glycolate uptake an extremely high background was apparent. This was caused by the build-up of $^{14}CO_2$ in stocks of ^{14}C-glycolate. Presumably this was due to

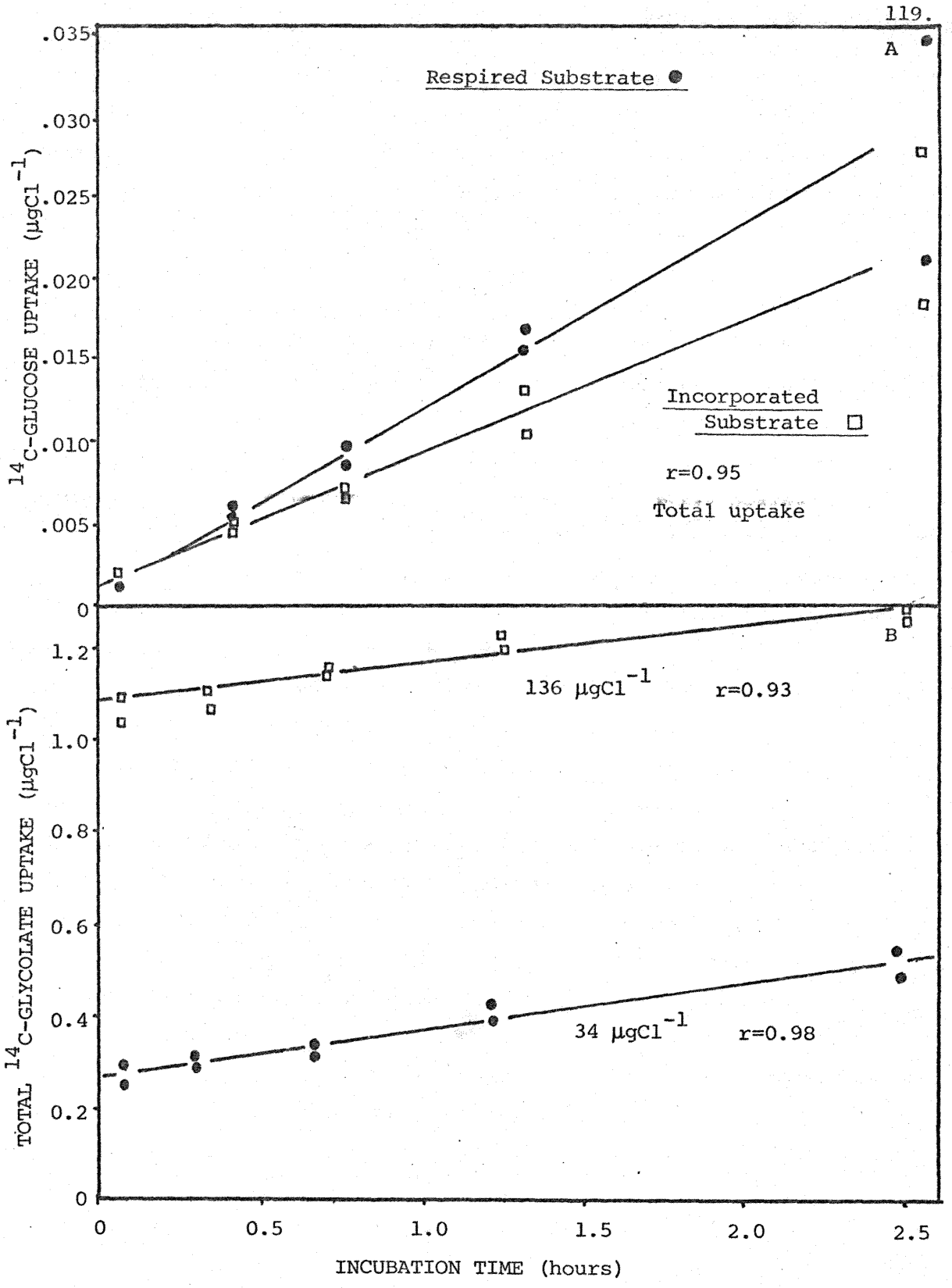


Figure 3.2/14 - Effect of Incubation Time on Substrate Uptake

Lake Rotomanuka

1400h 1.4.81

A - Glucose uptake at 2.1 µgCl^{-1}

B - Glycolate uptake at 34 µgCl^{-1} and 136 µgCl^{-1}

Incubation Temperature 20.0°C

chemical breakdown of the glycolate during storage as filtration of the ^{14}C-glycolate on to a 0.2µm membrane filter yielded insignificant activity on the filter. Acidification of the ^{14}C-glycolate followed by flushing with air reduced the $^{14}CO_2$ content to a level acceptable for use in heterotrophic uptake experiments.

3.2.4.5 Uptake of Glucose and Glycolate During the Period 3-4 January 1981

To obtain a large number of data points per curve and also to enable the use of both substrates, the sampling intervals were again increased to give two samples in 24 hours. Incubations were started in the laboratory (within one hour of sampling). The extra convenience afforded by the laboratory surroundings enabled extra incubations to be performed at each time. In fact 40 individual incubations were performed at each time. Filtrations were performed on one of each of the three replicate incubations at each concentration to provide data on the fraction of substrate incorporated.

Results using Glycolate

Results from both times of day are interpretable in terms of Michaelis-Menten kinetics (see figures 3.2/15 and 16). However, it is apparent that at both times uptake approached V_{max} over the entire concentration range. Given the scatter of data points that occurred, it is probable that the linearity of the modified Lineweaver-Burke plot is, in each case, merely a reflection of the fact that uptake remained almost constant with changing concentration. As uptake was constant, the value f, i.e. the fraction of added substrate which is taken up, decreased linearly with added substrate concentration and the graph of (incubation time/f) plotted against concentration was thus a straight line passing through the origin. As such it cannot be used to determine any of the kinetic parameters apart from V_{max}, which does not vary significantly between the two times of day.

Over the experimental period, natural glycolate levels were determined at each sampling time and indicated no significant change between the two times of day. The concentrations measured at 1400h and at 0100h were 52 µg of glycolate Cl^{-1} and 48 µg glycolate Cl^{-1} respectively. The 95% confidence intervals were not calculable from equation 4 in section 2.2.8 because of the shallow slope of the lines shown in figure 3.2/17 (Snedecor and Cochran, 1976), but it would be logical to assume that the two values are not significantly different. The

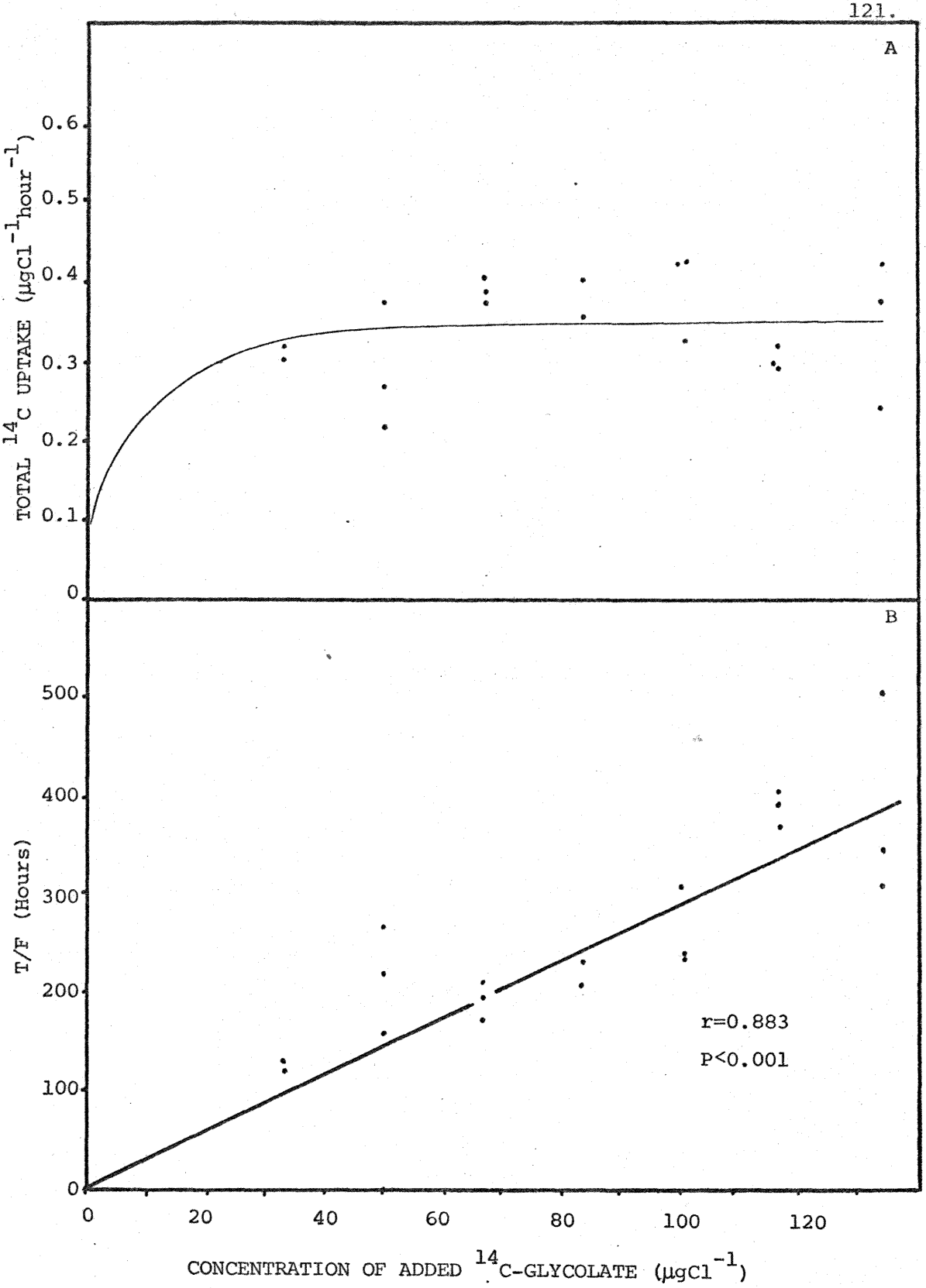


Figure 3.2/15-Uptake of ^{14}C-Glycolate in Lake Rotomanuka

1400h 3.4.81

A - Total uptake. Respired plus incorporated ^{14}C-activity

B - Incubation time/Fraction of added ^{14}C which was taken up

Incubation Temperature 21°C

Incubation Time 1hr 50min - 2hr 30min

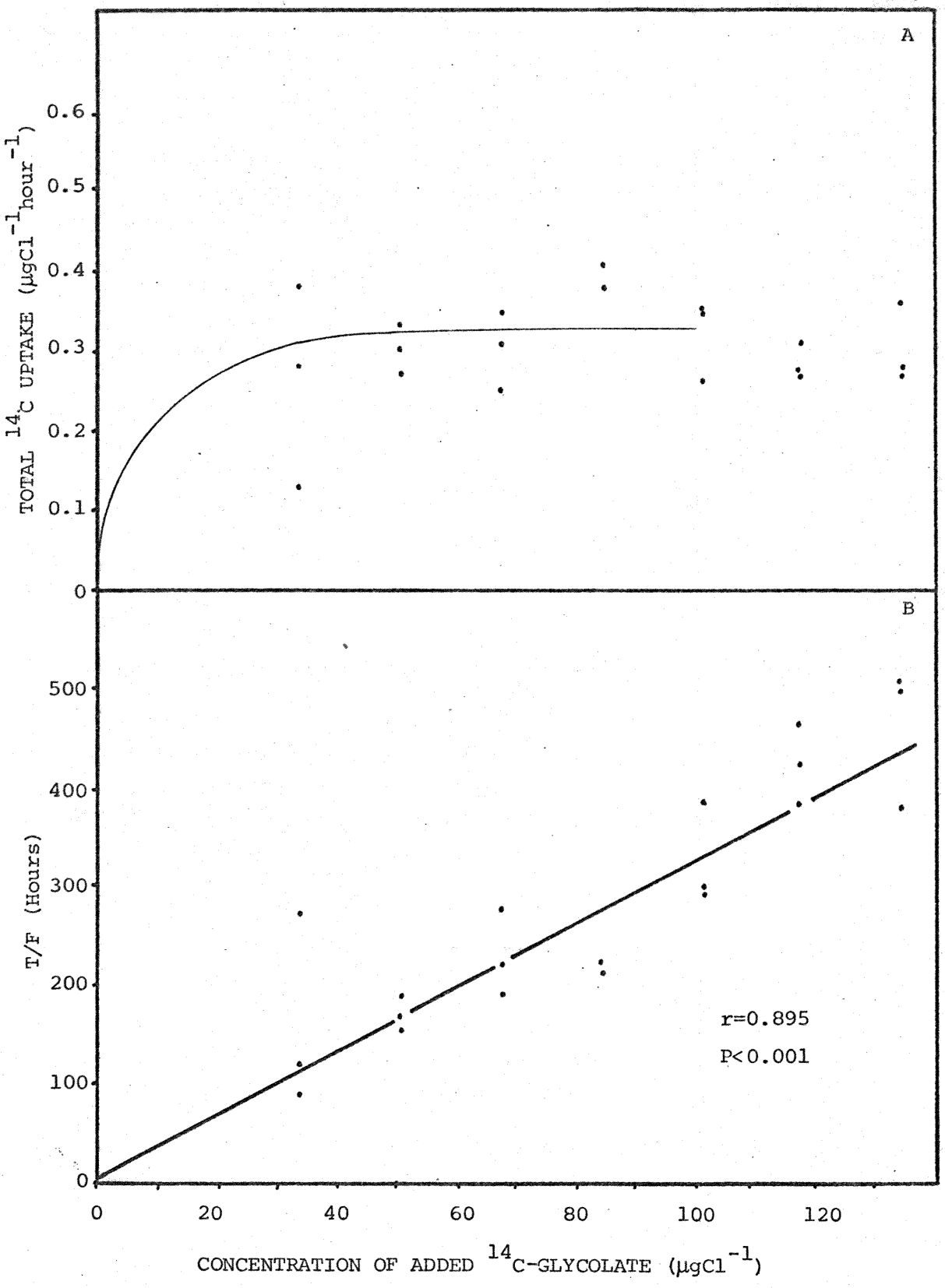


Figure 3.2/16-Uptake of ^{14}C-Glycolate in Lake Rotomanuka

0100h 4.4.81

A - Total uptake. Respired plus incorporated ^{14}C-activity

B - Incubation time/Fraction of added ^{14}C which was taken up

Incubation Temperature 20°C

Incubation Time 1hr 50min - 2hr 30min

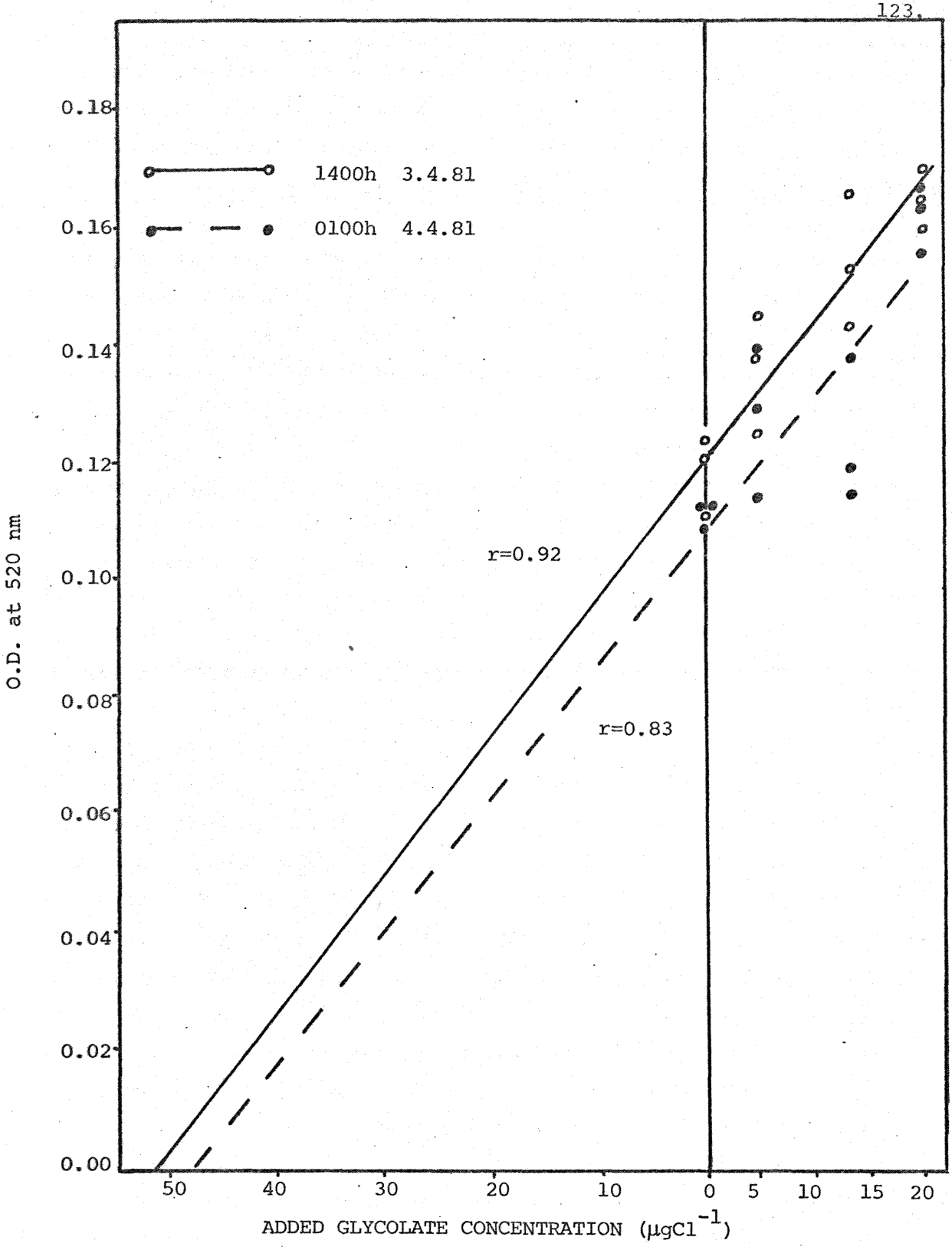


Figure 3.2/17 - Glycolate Analysis - Rotomanuka 3-4.4.81

Method of known addition, see section 2.6

Replicates are replicate assays of same extract with DHN

Results are: 1400h - 52μg glycolate carbon l^{-1}

0100h - 48μg glycolate carbon l^{-1}

measured glycolate values are high when compared with other values given in the literature. Wright and Shah (1975) found glycolate concentrations ranging from 0-26 μgCl^{-1} in coastal seawater at a variety of locations. Fogg, Burton and Coughlan (1975) found a range of 0-13 μgCl^{-1} in a variety of Antarctic lakes. Shah and Wright (1974) measured glycolate concentrations at a single marine coastal location over a period of one year and found a minimum of zero in winter and a maximum of 26 μgCl^{-1} in summer, coinciding with maximum phytoplankton populations. The high values measured in Rotomanuka on the 3rd-4th October 1980 (late summer) coincided with a bloom mainly consisting of the alga *Peridinium* (Pridmore, *pers. comm.*). The concentration of glycolate measured may have been sufficiently high to account for the apparent saturation of glycolate uptake systems at all concentrations of added glycolate. Measured glycolate concentrations of 48-55 μgCl^{-1} would suggest that the measured values of $(K_T + S_n)$ at 0100h and 1400h are meaningless as these were 5.4 and 2.9 $\mu gCl^{-1}h^{-1}$ respectively, implying that the K_T values were negative (see figure 3.2/20).

Results using Glucose

Both of the modified Lineweaver-Burke plots (see figures 3.2/18B and 3.2/19B) indicate a deviation from linearity similar to that seen in the plot for glycolate uptake from 1645h: 12/11/80 (see section 3.2.4.3), and explainable in the same terms. In this case it is possible to discount a respiration effect as suggested by Bell (1980) because there was no trend in the partitioning of substrate between incorporation and respiration with changing concentration of added substrate. Substrate depletion as a cause is also discounted because no more than 5% of added substrate was used in any case. Inhibition of uptake at higher concentrations of added substrate is very apparent, especially at 1400h on the 3rd of April. Inhibition seems to be the most likely cause of this particular deviation.

The effect of the increase in slope with increasing concentration of glucose in the modified Lineweaver-Burke plots is to yield negative values for the y-intercepts (T_t) and positive values for the x-intercepts ($-(K_T + S_n)$).

In an attempt to estimate more meaningful values for these kinetic parameters, the results obtained using the upper two concentrations

of glucose were arbitrarily ignored in each case. This approach gave values for V_{max} and T_t, which while significantly different from zero, were not significantly different from one another (see figure 3.2/21). Neither value obtained in this way for $(K_T + S_n)$ was significantly different from zero. On the basis of these results, it was not possible to demonstrate any change in the kinetics of uptake of glucose on a diurnal basis.

It could be argued, of course, that such an approach may or may not be valid, depending on whether the slope of the modified Lineweaver-Burke was continuously variable or, as was assumed here, linear below the concentration at which the apparent inhibition occurred. In view of the fact that it was not possible to demonstrate any diurnal change in uptake kinetics, arguments concerning the validity or otherwise of this approach are irrelevant in this instance.

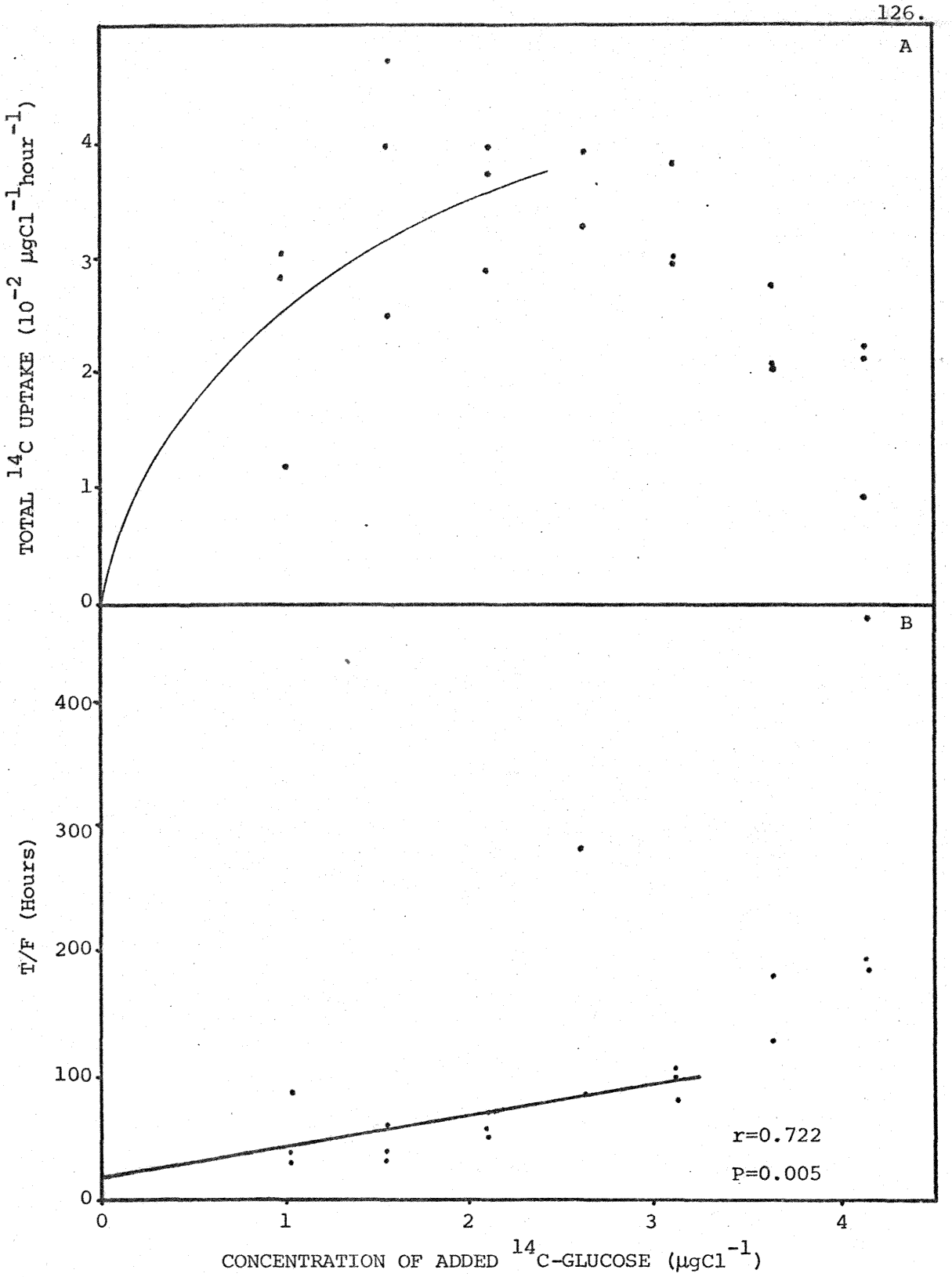


Figure 3.2/18 - Uptake of ^{14}C-Glucose in Lake Rotomanuka

1400h 3.4.81

A - Total uptake. Respired plus incorporated ^{14}C-activity

B - Incubation time/Fraction of added ^{14}C-activity which was taken up

Incubation Temperature 21°C

Incubation Time 1hr 50min - 2hr 20min

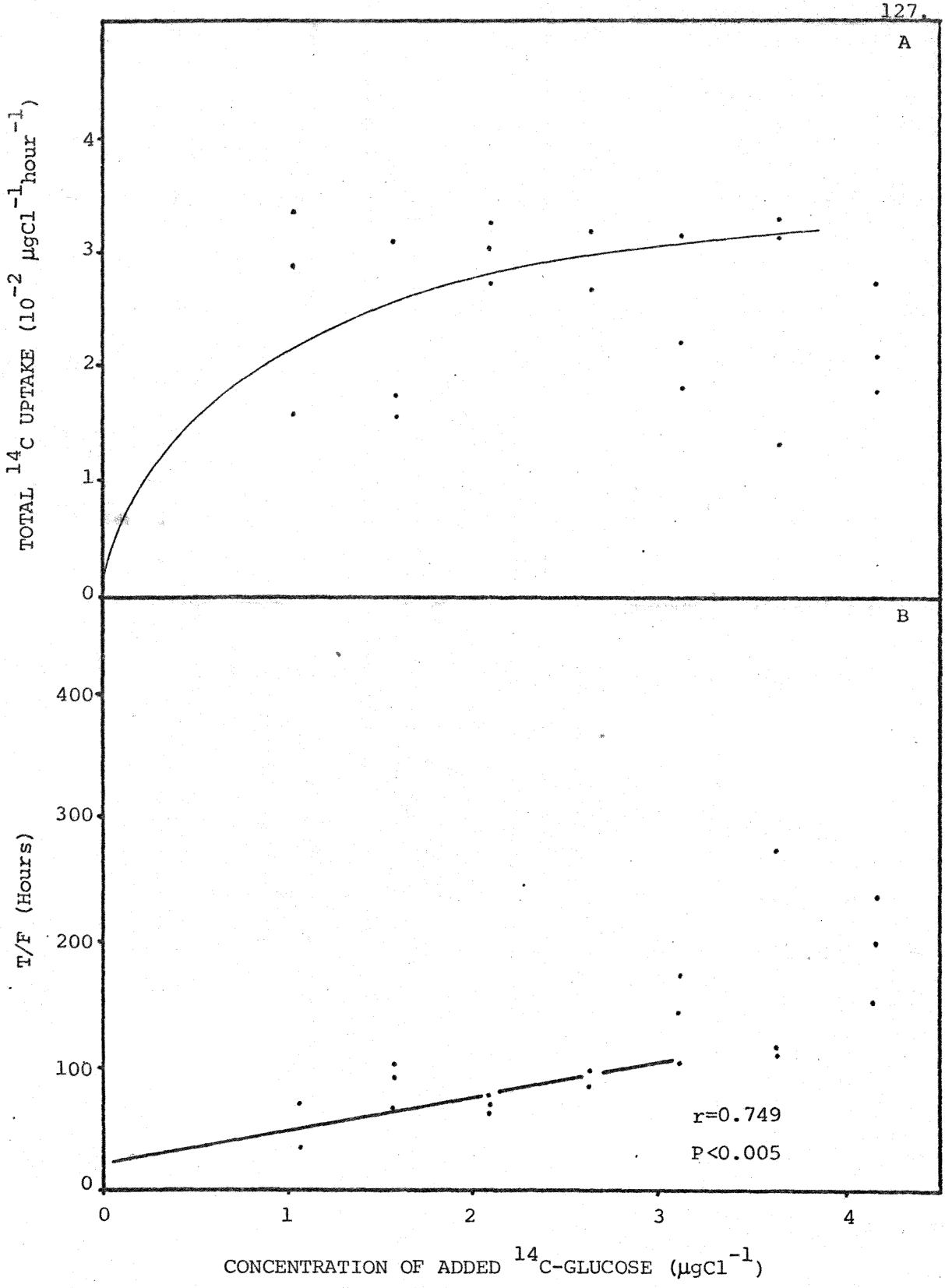


Figure 3.2/19 - Uptake of ^{14}C-Glucose in Lake Rotomanuka

0100h 4.4.81

A - Total uptake. Respired plus incorporated ^{14}C-activity

B - Incubation time/Fraction of added ^{14}C-activity which was taken up

Incubation Temperature 20°C

Incubation Time 2hr 0min - 2hr 50min

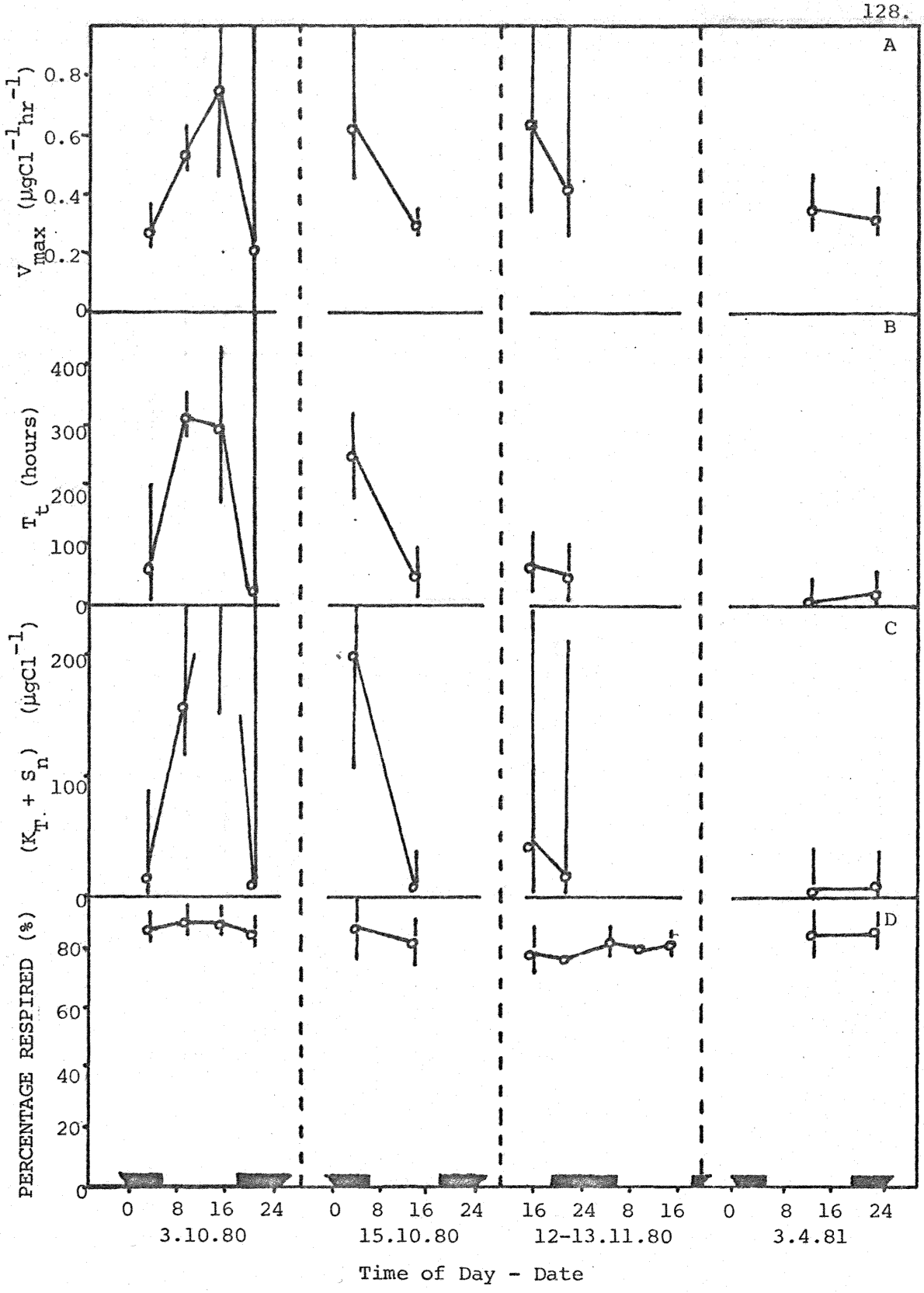


Figure 3.2/20 - Glycolate Uptake - Lake Rotomanuka

A - V_{max}; maximum uptake rate

B - T_t; turnover time

C - $(K_T + S_n)$; rate constant plus natural glycolate concentration

D - fraction of absorbed glycolate which was respired

Error bars show 95% confidence limits

sunrise sunset

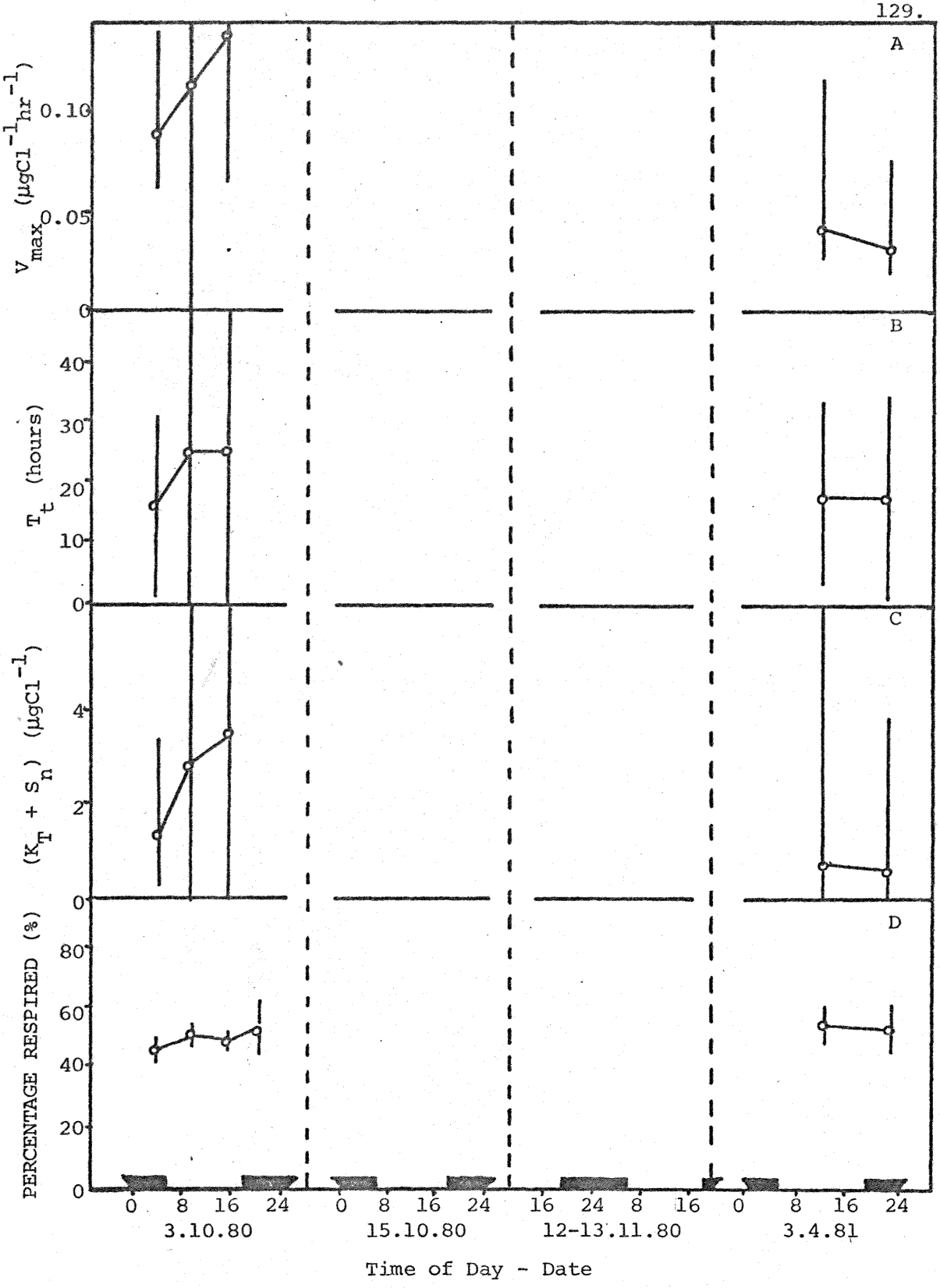


Figure 3.2/21 - Glucose Uptake - Lake Rotomanuka

A - V_{max}; maximum uptake rate

B - T_t; turnover time

C - $(K_T + S_n)$; rate constant plus natural glucose concentration

D - fraction of absorbed glucose which was respired

Error bars show 95% confidence limits

sunrise sunset

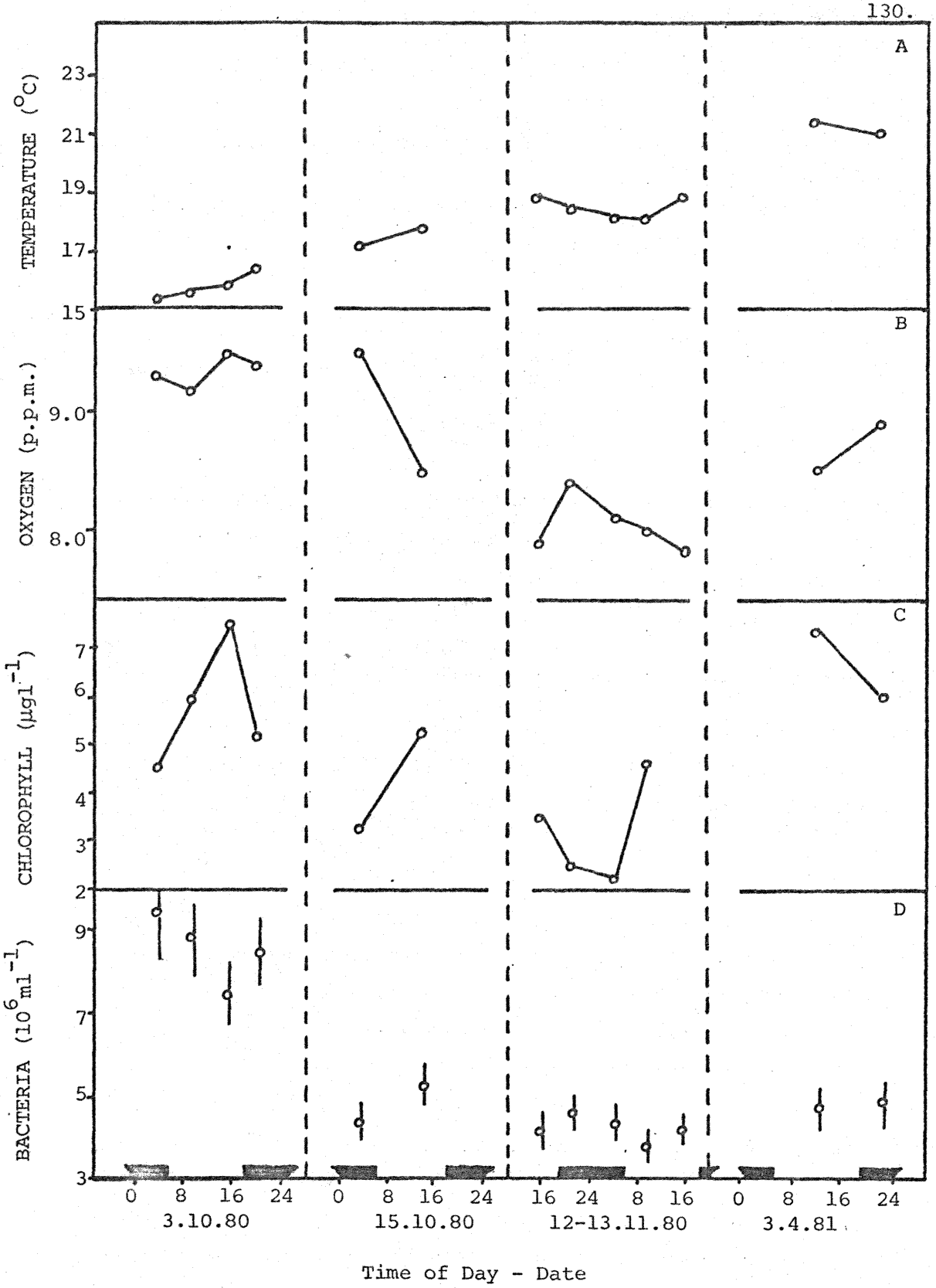


Figure 3.2/22 - Chemical and Biological Parameters - Lake Rotomanuka

A - Temperature at sampling depth (1m)

B - Oxygen concentration at 1m

C - Chlorophyll concentration at 1m (uncorrected for phaeophytin)

D - Bacterial direct count at 1m (error bars show 95% confidence interval)

sunrise sunset

3.3 GENERAL DISCUSSION

3.3.1 Range of Values Obtained for Measured Kinetic Parameters

(a) Glycolate

The ranges of the values of each of the measured kinetic parameters, V_{max}, $(K_T + S_n)$ and T_t were as follows:

V_{max} ranged from 0.29 to 0.81 $\mu gCl^{-1}hr^{-1}$

$(K_T + S_n)$ ranged from 23 to 370 μgCl^{-1}

T_t ranged from 25 to 322 hours.

These values are all estimated from modified Lineweaver-Burke plots which meet the acceptibility criteria given in section 2.2.8 (i.e.$<P$ .05). The values of $(K_T + S_n)$ and T_t from 3-4/4/81 have been excluded because of the uncertainty about their validity (see section 3.2.4.5).

All of the above values are within the range of values reported by Wright and Shah (1975) for the uptake of glycolate from a variety of marine, coastal environments. The measured values for T_t were all greater than 24 hours, although in some cases the lower confidence limits tended to zero. It is difficult to draw conclusions from this, on the effect of glycolate turnover on the supply of glycolate on a diurnal basis.

It is interesting to note that the extremes of the range of values of V_{max} were both measured on the same day (3/10/80). This result is of significance to workers using kinetic parameters to observe seasonal trends in lakes. In this study seasonal trends were masked by diurnal variations in uptake. The results of 12-13/11/80 suggested that excluding diurnal variation from the results of seasonal sampling programmes might be difficult. On this date the observed diurnal variations did not follow a diurnal cycle in that completely different uptake kinetics were observed at the same time on two adjacent days. Thus, when comparing uptake at different times of year or similarly in different water bodies, a sampling programme involving a single sample per day at a fixed time of day may not be sufficient to exclude the effects

of short term variations in uptake kinetics. This finding is similar to the finding of Gocke (1975) who found considerable short term (<24 hours) variation in the uptake of glucose and acetate in a marine fjord. Further, it extends the need to consider short term variation as a source of experimental error in sampling programmes involving bodies of water which are not as obviously heterogeneous as the fjord studied by Gocke. Gocke also measured short term changes in salinity, temperature, bacterial colony forming units and ammonia and phosphate concentrations, and suggested that the observed variations in these and in substrate uptake were due to sampling from a site where different water masses were meeting and mixing. Although such large scale effects seem unlikely in Lake Rotomanuka and were certainly not indicated by diurnal changes in either bacterial numbers, temperature or oxygen (see figure 3.2/22), small scale patchiness in the distribution of active heterotrophs might have been the cause of the observed variation in uptake. Bacterial numbers did not change significantly between different times of day on any of the sampling dates. Temperature and oxygen concentration varied over the whole depth profile on each sampling date, but the changes seen were typical of normal diurnal changes in these parameters, with oxygen and temperature decreasing at night (Wetzel, 1975), rather than being random changes which would be associated with mixing of water masses. Furthermore, the temperature and oxygen profiles of Lake Rotomanuka (figures 3.2/2 and 3) indicate that the lake is generally well circulated for at least the first two metres.

The problem of spatial heterogeneity in the distribution of bacteria in lakes was investigated to some extent by Palmer, Methot and Staley (1976) who found, using conventional plate-counting techniques, that the distribution of colony-forming units within a lake exhibited considerable patchiness.

(b) Glucose

Only one sample (0400h: 3/10/80, see figure 3.2/5) produced an acceptable modified Lineweaver-Burke plot when all available data points were used. By ignoring the acceptibility criteria ($P<0.05$), and by ignoring data points obtained from the two

highest concentrations of added glucose on 3-4/4/81 (see section 3.2.4.5), it was possible to derive kinetic parameters from five out of the six available plots. However, the 95% confidence limits of these parameters were so wide that it was not possible to find any significant difference between any of the values of either V_{max}, T_t or $(K_T + S_n)$ (see figure 3.2/20).

Ignoring the confidence limits of the results, the ranges of values for each parameter were:
V_{max} ranged from 0.018 - 0.14 $\mu gCl^{-1} hr^{-1}$
$(K_T + S_n)$ ranged from 0.6 - 3.7 μgCl^{-1} and
T_t ranged from 17-33 hours.

All of these values are within the ranges of previously measured values for glucose uptake in different water bodies throughout the world (see Hobbie and Rublee, 1977; and Wright and Hobbie, 1966) and in different lakes in New Zealand (Gillespie, 1976).

3.3.2 Variability of Replicates

At all times when replicate incubations were performed to determine the uptake rates of either glucose or glycolate, considerable variation in results was observed between replicates containing the same concentration of added substrate. See, for example, figures 3.2/7 and 3.2/8.

The first aspect of the work to examine for sources of variability is experimental technique, particularly the efficiency of recovery of ^{14}C as $^{14}CO_2$ or as incorporated ^{14}C on a filter. However, this source of error only accounted for some of the observed scatter of points about the graphs in figure 3.2/7. Variability in either method of recovery would be seen as variability in the measured percentage of ^{14}C respired. In figure 3.2/8 in which $\frac{(\text{incubation time})}{(\text{fraction of glucose take up})}$ is plotted against concentration of glucose, for the experiment done at 1540h on 15th October 1980, the residual root mean square of the linear regression is ± 97 hours. This is a measure of the scatter of points about the line at the mean concentration of added substrate. The coefficient of variation of the percentage of glycolate respired at 1540h (based on all 20 data points) was 5%, which would give a scatter about the line of only ± 25 hours at the

mean. Comparison of these two standard errors using an F-test shows that they are significantly different at the 1% level of probability. There is a similar difference between the scatter of points about the plot of figure 3.2/8B for 0430h in the same experiment, and the standard deviation of the mean percentage respired at 0430h. Thus, assuming that the plots in both figures 3.2/7B and 3.2/8B are adequately represented by straight lines, the scatter about the lines is not solely, or even largely due, to variability in the recovery of ^{14}C. Neither is it due to other obvious experimental errors, such as pipetting errors or ^{14}C-counting errors which were each responsible for only about ± 1% variation.

Other workers in the heterotrophic uptake field have commented on the scatter of results from replicate incubations. Wright and Shah (1975) had differences of up to 100% between replicate samples when using glycolate as a substrate. No reason was given. Wright (1971) suggested that such variability between replicates was caused by spatial heterogeneity of heterotrophs within the sampled water. This heterogeneity was a result of the adherence of active heterotrophic bacteria to much larger particles of detritus or to algal cells. This phenomenon of absorbance was observed in my samples, using epifluorescence microscopy and resulted in the need for ultrasonication of samples in order to break up clumps of bacteria before direct counts were made (see section 2.3). Thus, although replicate samples for incubation were taken from a well-agitated bulk water sample of 2.5 litres, it is possible that the presence of small numbers of detrital particles with associated highly active heterotrophic bactiera, within this bulk sample, could have resulted in significantly different numbers of heterotrophically active bacteria being drawn into replicate 10ml subsamples.

A second possible source of the observed variability between replicates is the retention of substrate on the walls of the syringes used for incubation. No attempt was made to determine whether the washing procedure used (see section 2.2.7) removed all of the substrate used in previous incubations. This point is particularly relevant in view of the fact that the concentrations of substrate which were used to rinse the incubation syringe and filter were up to one million times higher than the concentration of labelled substrate used to measure uptake.

3.3.3 Constancy of Percentage of Carbon Respired as CO_2

For both glucose and glycolate, partitioning of the substrate into the biosynthetic and the respiratory pathways after uptake, remained constant, within experimental error, over each 24 hour sampling period and also over the sampling season (see figures 3.2/20 and 21). This observation could be explained in one or more of three ways: (i) the population of heterotrophs which utilised each substrate remained constant in composition throughout the sampling period or; (ii) the partitioning of a given substrate was the same, or similar, within a range of organisms, under different conditions or; (iii) some variation in the percentage respired did occur but was masked by experimental error. On 3/10/80 the percentage respiration of glycolate appeared to increase as V_{max} increased and the percentage respiration of glucose appeared to increase during the summer months.

No further evidence was obtained to support any particular explanation. The average percentage respiration of glycolate was 84% and that of glucose was 52%.

3.4 SUMMARY

(a) Changes in the uptake kinetics of glycolate were observed over three out of four 24 hour periods. On two dates, these changes were observed as significant changes in measured kinetic parameters. On one date they were observed as changes in the relationship between substrate uptake rate and concentration of added substrate, but the changes were not quantifiable.

(b) Apparent but unquantifiable changes in the uptake kinetics of glucose were observed over one out of two 24 hour periods. These changes were observed as changes in the relationship between substrate uptake rate and concentration of added substrate, at the upper end of the range of added substrate concentrations.

(c) The diurnal changes in the uptake kinetics for glycolate did not follow a constant pattern and on the one occasion where the variation in kinetics was followed over a full 24 hours the pattern of variation was not cyclic within that 24 hours.

(d) Because of the large errors involved in determining glycolate turnover times, it is not possible to make conclusions about whether heterotrophic utilisation of glycolate is likely to alter glycolate concentration on a diurnal basis. On some occasions the turnover times were considerably greater than 24 hours. Turnover times were never significantly less than 24 hours.

(e) On the one 24 hour sampling period for which natural glycolate concentrations were successfully measured, no change in glycolate concentration occurred between the two sampling times (3-4/4/81). Although, on the dates that these measurements were made, it is possible to conclude that diurnal changes in extracellular release or glycolate uptake have <u>not</u> been responsible for changes in glycolate concentration, it is not possible to make similar conclusions for the other sampling periods.

(f) No conclusions could be drawn regarding the causes of the diurnal variations in uptake kinetics observed. The three possibilities (and these are not mutually exclusive) are:

(i) Heterogeneity in the distribution of heterotrophs within the sampled water body.

(ii) Physiological or population adaptations by heterotrophs.

(iii) The effects of sampling and incubation conditions on uptake kinetics.

(g) It is important to consider the effects of short term changes in uptake kinetics when using uptake kinetics for comparative purposes.

(h) The fraction of each substrate which was respired after uptake remained constant throughout each sampling period and over the sampling season.

Chapter 4

LAKE FRYXELL

RESULTS AND DISCUSSION

4.1 INTRODUCTION

During the summer of 1980/81 I was presented with the opportunity to repeat some of the work which had been started on Lake Rotomanuka, on a small ice-covered Antarctic lake under a regime of continuous sunlight (24 hours per day). It was felt that a study of possible diurnal changes in heterotrophic uptake kinetics under such conditions would make an interesting comparison with a similar study made under the more conventional lacustrine conditions found in Lake Rotomanuka. Any results from such a study would also add to the scanty body of knowledge on mainland Antarctic lakes.

The following study was conducted as part of a continuing programme of research by the University of Waikato Antarctic Research Unit.

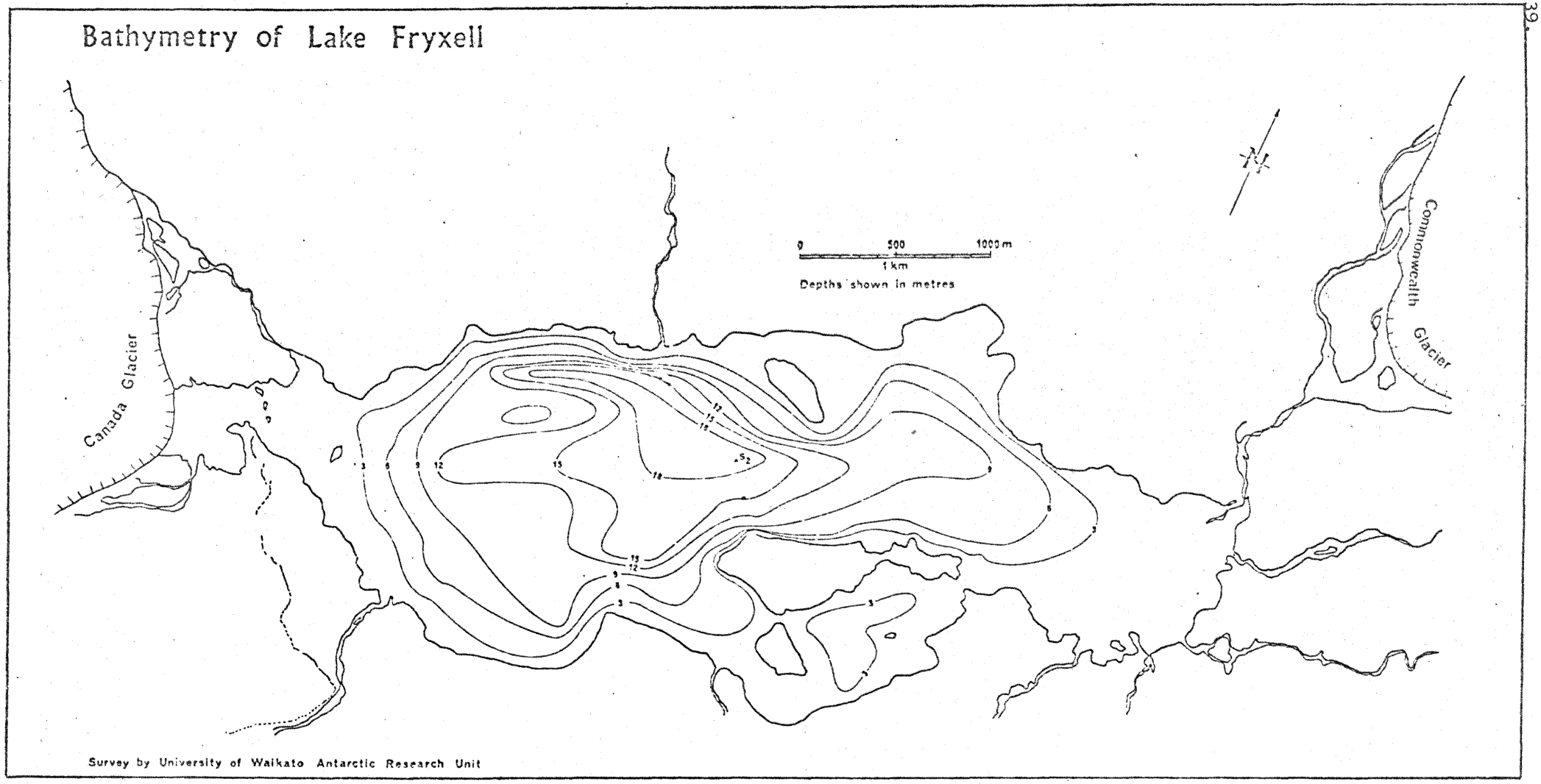


Figure 4.2/1 - The Bathymetry of Lake Fryxell showing sampling site (S_2)

4.2 LAKE FRYXELL - DESCRIPTION

Lake Fryxell (77°37'S, 163°10'E) is situated in the lower Taylor Valley, South Victoria Land, Antarctica, at an altitude of 22m. The lake covers an area of 7 km^2 and has a maximum recorded depth of 18.5m from the water surface. The lake surface is permanently covered by up to 4.5m of ice, except for a 'moat' of 1-10m of open water which appears around the shore over several months in summer. The water column, being protected from the wind, contains permanent temperature and salinity gradients with chloride concentration ranging from 1,000 mgl^{-1} beneath the ice surface to 4,200 mgl^{-1} at 18m (Hoare *et al*, 1965). The lake has temporary inflows, consisting of melt-water from the Canada and Commonwealth glaciers in summer, but no known outflow.

Profiles of oxygen, temperature, chlorophyll *a*, bacteriochlorophyll and bacterial direct counts, based on my own data, are shown in figure 4.2/2.

4.2.1 Sampling Site and Sampling Procedure

The sampling site coincided with the mid-lake station used by Vincent (1980) (see figure 4.2/1). A sampling hole was made in the ice with a 10cm diameter SIPRE ice auger. The hole was covered with a polar tent to prevent refreezing and to protect samples from the elements. Samples were collected through a 6mm i.d. p.v.c. tube into an evacuated flask. Vacuum was supplied by a Nalgene hand vacuum pump (Nalge Company). A diffuser, consisting of two 6cm diameter plastic discs held 1cm apart by spacers, one penetrated by the end of the tube and the other below the opening of the tube, was used to ensure sampling was from a defined depth. The tube was weighted and left in place permanently to reduce the effects of initial stretch and kinking on the sampling depth. A volume, equal to the volume of the tube was discarded between samples. All depths are expressed as depth in metres from the water level within the sampling hole. For heterotrophic uptake experiments a bulk 2.5 litre sample was taken and stored in the dark for return to the camp. Incubations were started within 1½ hours of sampling.

4.2.2 Diurnal Variation in Solar Radiation at Ice Surface

Samples were taken during the summer of 1980/81 at a period when the sun was above the horizon for 24 hours a day. During the sampling

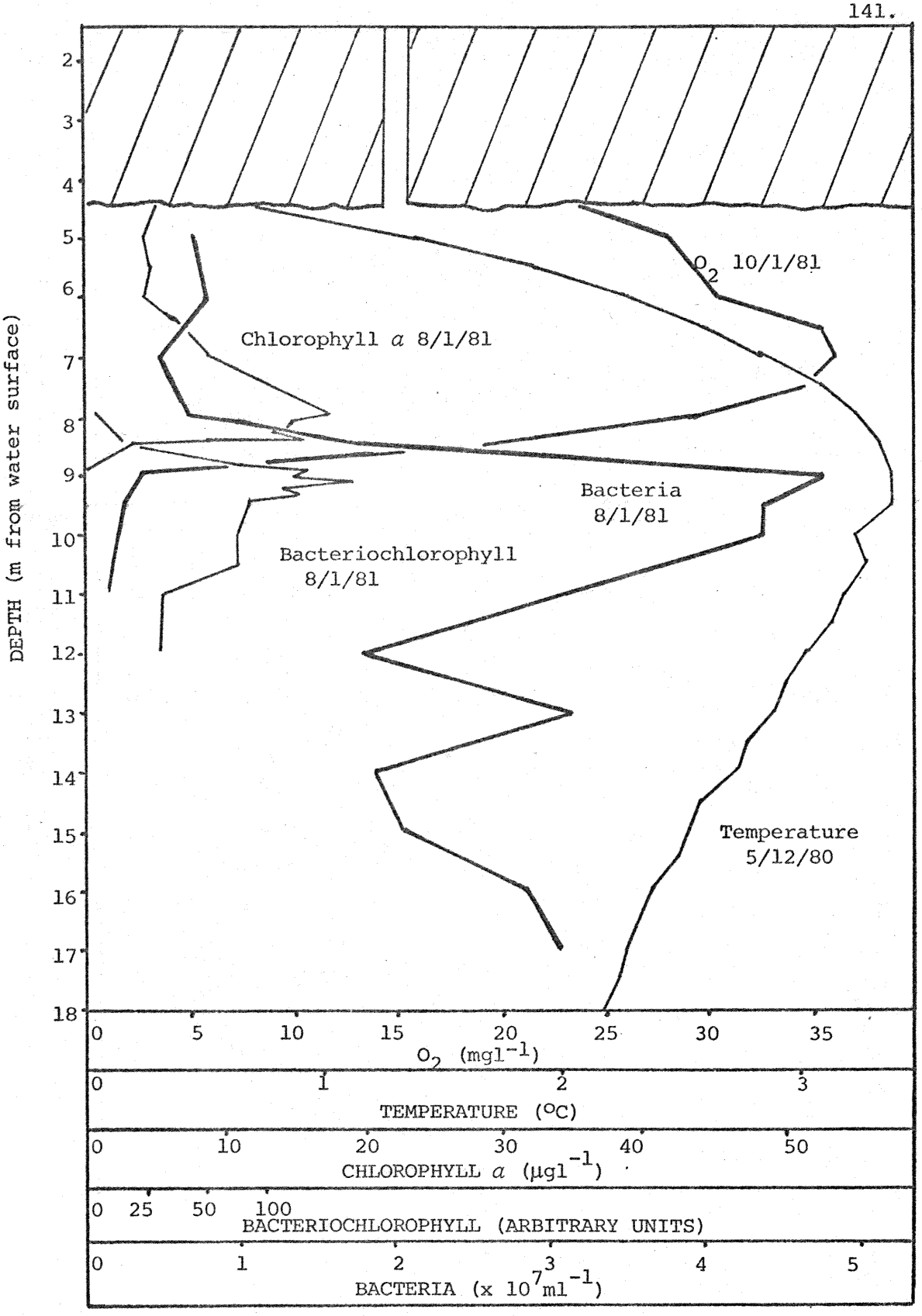


Figure 4.2/2 - Biological and Chemical Profiles - Lake Fryxell

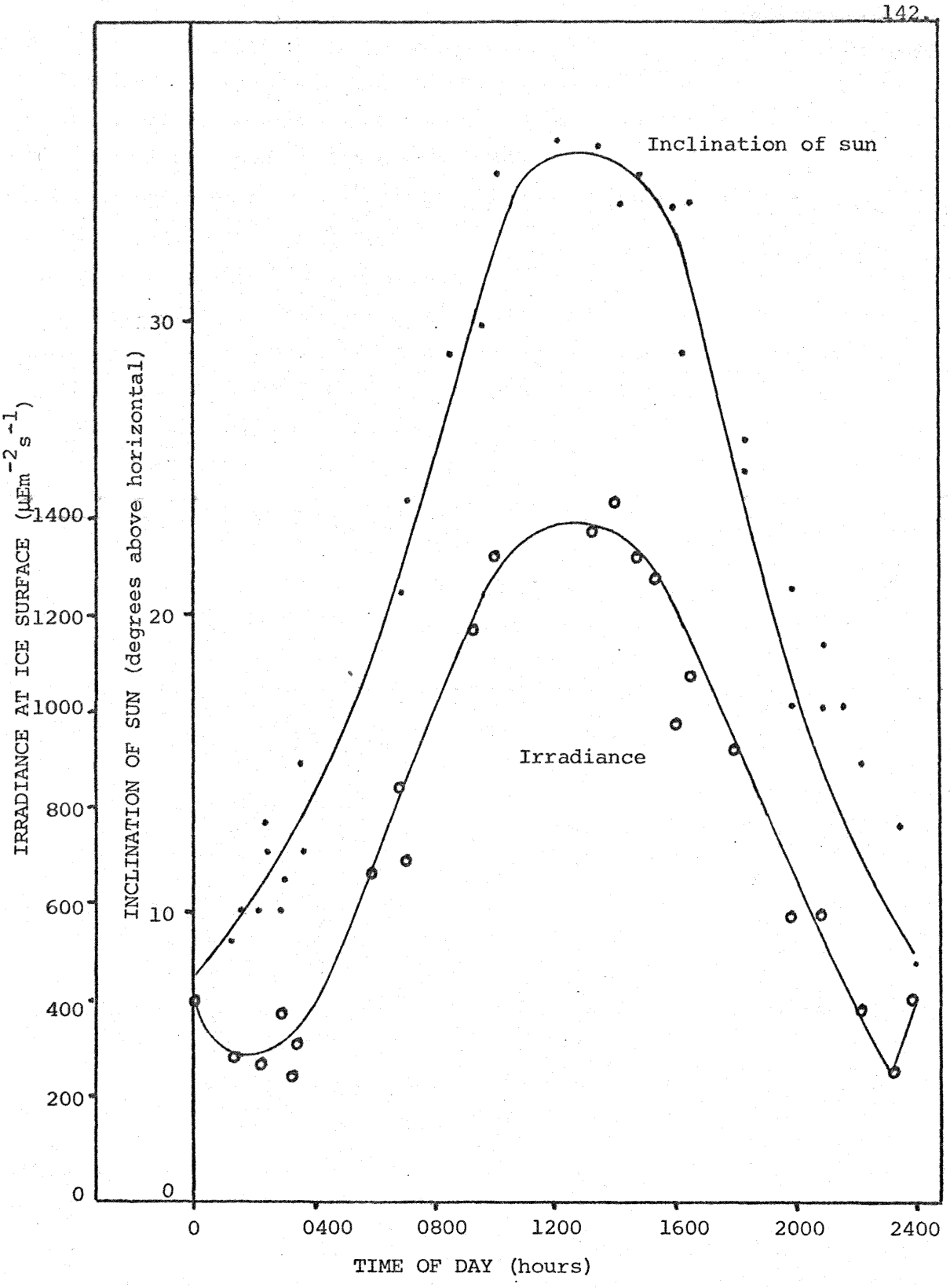


Figure 4.2/3 - Inclination of Sun and Irradiance at Ice Surface
Lake Fryxell - January 1981

The two minima in illuminance at 0300h and 2330h are due to partial eclipsing of the sun by mountain peaks

period the inclination of the sun was followed throughout the day with an inclinometer to give figure 4.2/3. This variation in solar inclination resulted in a 5.8-fold variation in irradiance at the ice surface which ranged from approximately 1,450 $\mu Em^{-2}s^{-1}$ to approximately 250 $\mu Em^{-2}s^{-1}$ on a clear day (figure 4.2/3) (see section 2.8). Without taking into account the effect of solar angle on the loss of light by reflection the variation in illumination below the ice must have had at least a 5.8 fold range throughout each 24 hour period. Whether this would have been high enough to affect extracellular release was not known.

4.3 PRELIMINARY INVESTIGATION AND ASSESSMENT OF ANALYTICAL PROCEDURES

4.3.1 Introduction

Work on Lake Fryxell the previous summer, by Harfoot (*pers. comm.*) and Vincent (1980) showed that the stable stratification in Lake Fryxell has led to microstratification of the microbial inhabitants of the lake. In general terms, this microstratification involves several distinct peaks of algal activity in the photic zone, which reached from the lower surface of the ice, at 4.5m, to about 9m. Below 9m a sharp oxycline and a marked reduction in incident light inhibit algal growth. The greatest algal population, consisting chiefly of motile phytoflagellates, including cryptomonads, is found immediately above the oxycline. Bacterial numbers in the photic zone are associated with peaks of algal activity. An increase in numbers and cell size in the oxycline is responsible for the largest peak in bacterial biomass at about 9m. Bacterial biomass then decreases to a minimum at 15m and increases again as the sediments approach. Vincent (1980) found some evidence that algal migration occurred at night.

My initial work consisted of relocating peaks of microbial activity and identifying a suitable sampling depth for studies of heterotrophic uptake on a diurnal basis. Suitability, in this context implies (i) maximum heterotrophic activity and (ii) lack of diurnal migration. Because of the low water temperature in Lake Fryxell (0-3.5°C), maximum activity was considered to be an imperative to keep heterotrophic incubation times short.

The techniques used at this stage were the ETS technique (section 2.5), chlorophyll extraction (section 2.4) and a preliminary heterotrophic uptake experiment.

4.3.2 Preliminary Findings

4.3.2.1 Preliminary Heterotrophic Uptake Results

In the preliminary uptake experiment water samples from 6m (photic zone), 9m (transition zone), and 12m (euphotic zone) were incubated in the dark, for various lengths of time, with ^{14}C-glucose or ^{14}C-glycolate at added concentrations of 2.2 μgCl^{-1} and 74 μgCl^{-1} respectively. The generous co-operation of the U.S. National Science Foundation enabled filters and trapped CO_2 to be counted in a liquid scintillation counter (Beckman LS100C) at McMurdo base. Because of a limitation on counting

time, suitable blanks were not counted and the counting efficiency of the machine was not assessed. Nevertheless the results indicated:

(i) at 6m glycolate uptake was negligible, even after 18 hours incubation, while glucose uptake was measurable after six hours;

(ii) at 12m glucose uptake was negligible, even after 18 hours incubation, while glycolate uptake was significant;

(iii) at 9m, both glucose and glycolate were taken up in measurable quantities;

(iv) in each case where a substrate was taken up the absolute uptake increased with time although linearity of this relationship could not be tested without formalinised blanks;

(v) the maximum uptake rate for glucose was at 9m. At 12m the uptake of glycolate was only marginally faster than at 9m.

It is noteworthy that glycolate, which I have assumed to occur in natural waters as a result of algal release, is not taken up in the photic zone but is in the euphotic zone, where it would be less likely to be found, and glucose, which I have assumed to be a decomposition product is utilised in the photic zone but not in the euphotic zone, where it would be more likely to be available normally. This result is opposite to that of Wright (1968) who found that, in a temperate-zone lake glycolate uptake decreased with depth whilst glucose uptake increased with depth.

4.3.2.2 Chlorophyll *a*

Examination of the chlorophyll *a* profile in figure 4.3/1 reveals an apparent trend in which the peak of chlorophyll *a* at 8.4m is broader at mid-day than at midnight. At midnight on 24/12/80 and 8/1/81 a second peak is visible above the 8.m peak. At midnight on 30/12/80 the 8.4m peak has moved upward. Unfortunately, due to limitations on the number of samples which could be processed at one time, several of the profiles do not extend up far enough to be conclusive. However, the implication of these results is that some of the motile algae situated between 8.0 and 8.5m migrate upwards at night. Although, the magnitude of this migration is possibly as little as 25cm the increase in light achieved by such a migration is in the order of 20%. This figure is based on work done the previous summer by the University of Waikato Antarctic Research Unit, in which it was found that the

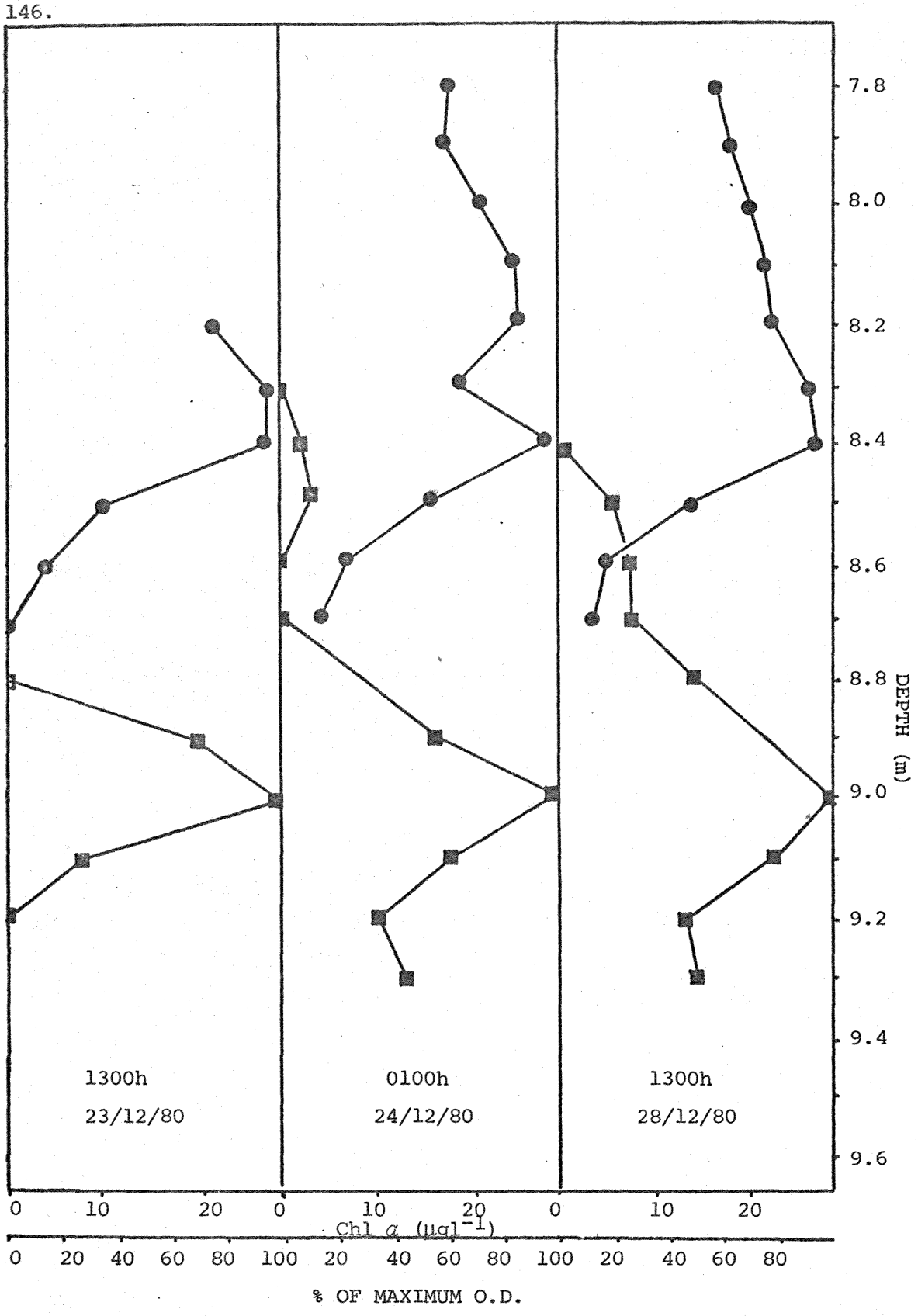
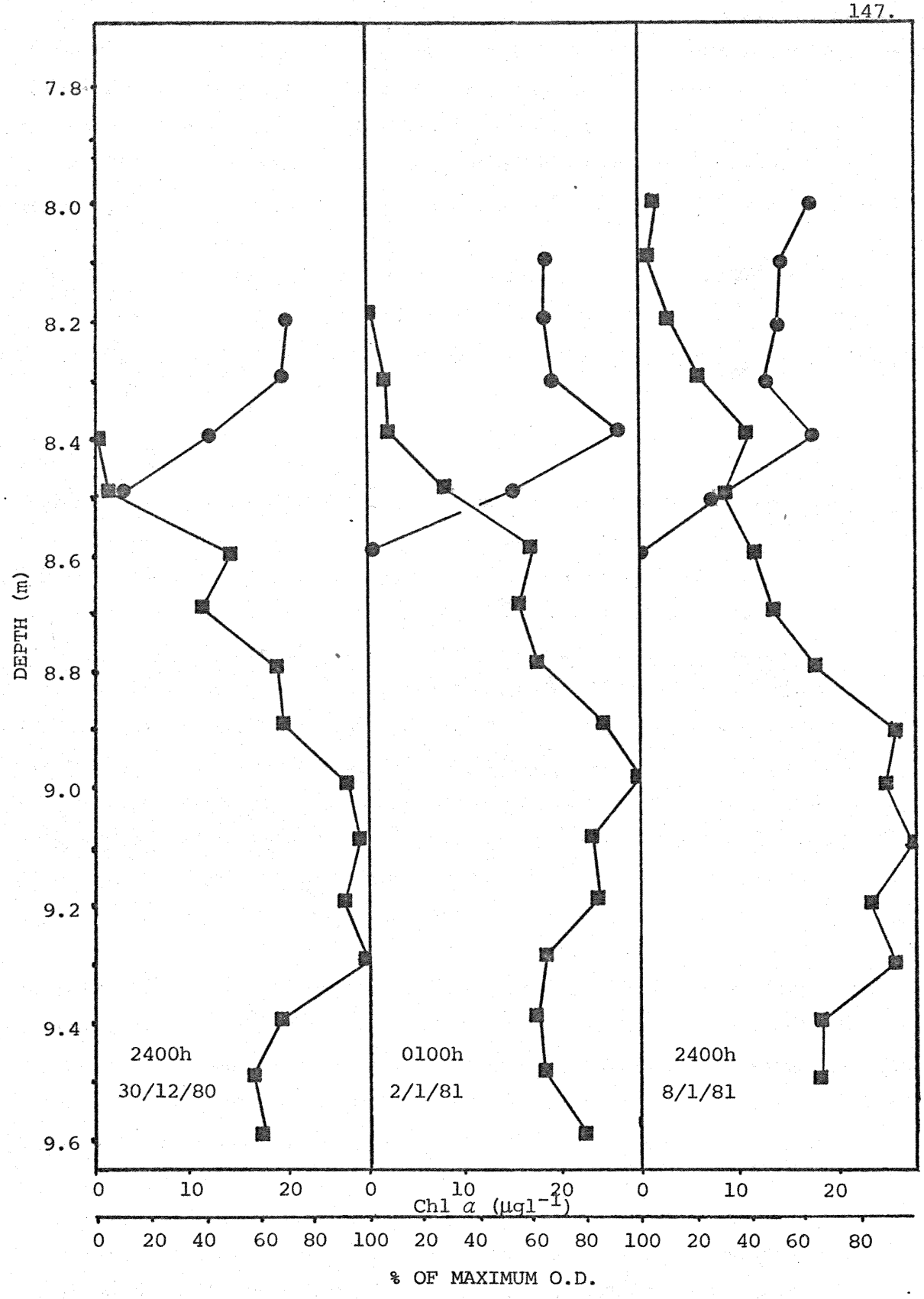


Figure 4.3/1 - Chlorophyll *a* and Bacteriochlorophyll vs. Depth
Lake Fryxell

The upper plot is chlorophyll *a* (μgl^{-1})

The lower plot is bacteriochlorophyll (% of maximum O.D.)


DEPTH (m)
7.8
8.0
8.2
8.4
8.6
8.8
9.0
9.2
9.4
9.6
2400h
30/12/80
0100h
2/1/81
2400h
8/1/81
0
10
20
Chl a (µgl⁻¹)
0
20
40
60
80
100
% OF MAXIMUM O.D.

irradiance (photosynthetically active radiation) in Lake Fryxell increased from 1.2 μEm^2s^{-1} (0.39% of surface irradiance) at 8.5m to 1.7 μEm^2s^{-1} (0.55% of surface irradiance) at 8.0m (Harfoot, *pers. comm.*).

A small diurnal migration between 8.0 and 8.5m is consistent with the results of Vincent (1980) which showed a broadening of the chlorophyll peak between 8.0 and 8.5m at night when chlorophyll concentrations were measured at 0.25m intervals.

4.3.2.3 Bacteriochlorophyll

Using the hot methanol extraction technique, a band of reddish purple pigment, absorbing maximally at 780nm was discovered at 9m. This is shown in figure 4.3/1 along with the chlorophyll *a* results, and is expressed as a percentage of the peak value of the term ($O.D._{780}$ - $O.D._{810}$) at each sampling date. It was necessary to use 810nm to correct for non-pigment absorption because of the absorption maximum of the pigment near the traditionally used 750nm. Expressing the results as a percentage was necessary because the extreme photolability of the pigment was not realised during extractions prior to 30/12/80. After it was discovered that the $O.D._{780}$ of extracts was reduced by as much as 80% after one minute of exposure to sunlight, all extractions were performed in test tubes wrapped in tin foil and capped.

Probable source of the reddish purple pigment is bacteriochlorophyll *a* contained in photosynthetic bacteria. This pigment is known to have an absorption maxima at 772nm in methanol extracts (Dawson *et al*, 1969). Photosynthetic bacteria were found at the oxycline of another Antarctic lake by Hand and Burton (1980).

No pattern of movement on a diurnal basis could be detected for the bacteriochlorophyll peak. If diurnal migrations by photosynthesisers are primarily to achieve a balance between limiting light, which decreases with depth, and nutrient availability, which increases with depth, then non-migration by this band of photosynthetic bacteria suggests either (i) inability to migrate, or (ii) constant light limitation (nutrient excess) with some constraint on further upward movement. Possible constraints are O_2 excess and H_2S depletion. H_2S was first detectable by smell in water samples at 8.5-8.6m.

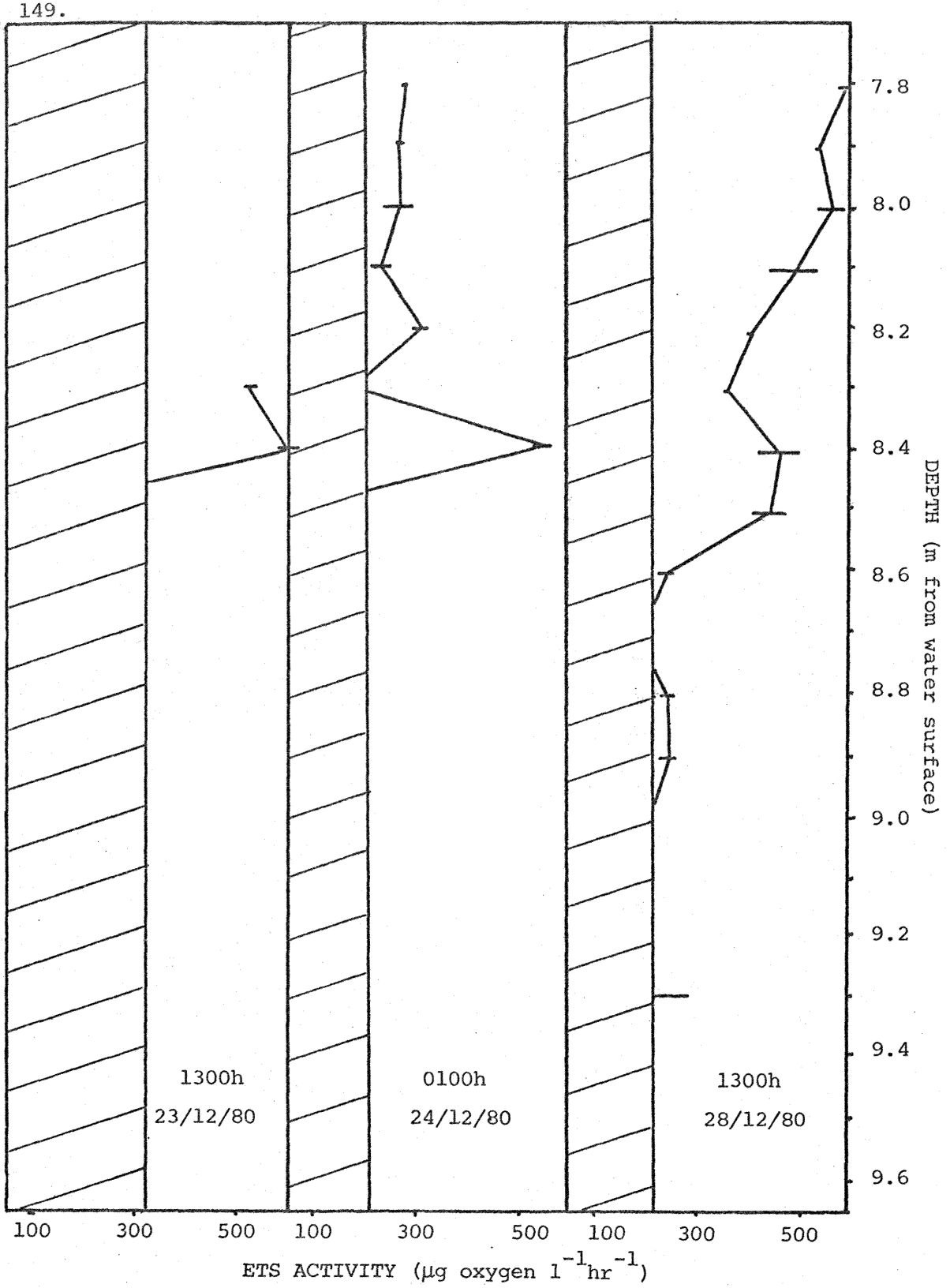
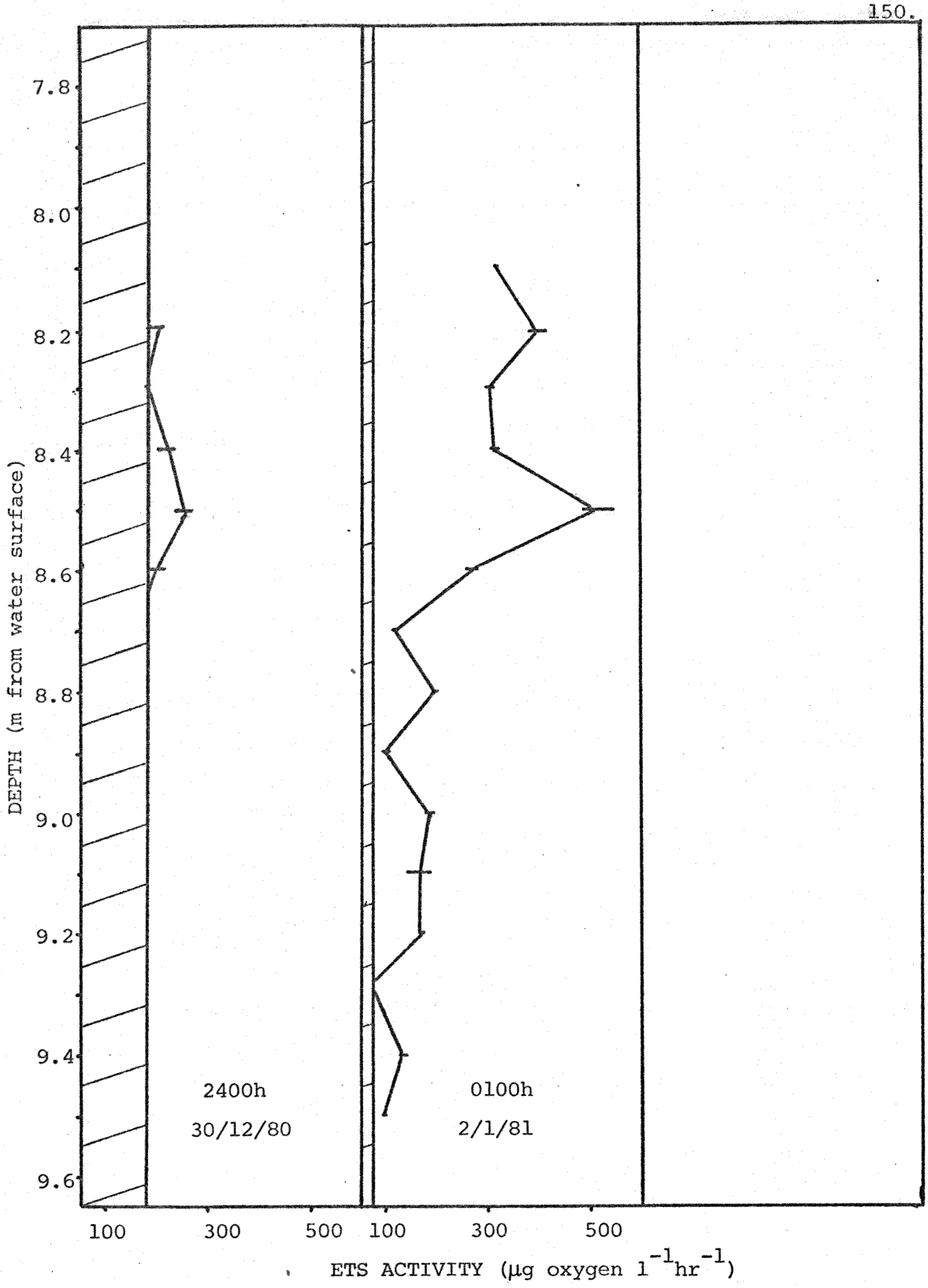


Figure 4.3/2 - ETS Activity vs. Depth - Lake Fryxell

Bars indicate range of duplicate assays on same enzyme extract

Shaded region is less than lower limit of detection (see section 2.5(e))


7.8
8.0
8.2
8.4
8.6
8.8
9.0
9.2
9.4
9.6
DEPTH (m from water surface)
2400h
30/12/80
0100h
2/1/81
100
300
500
100
300
500
ETS ACTIVITY (µg oxygen l-1hr-1)

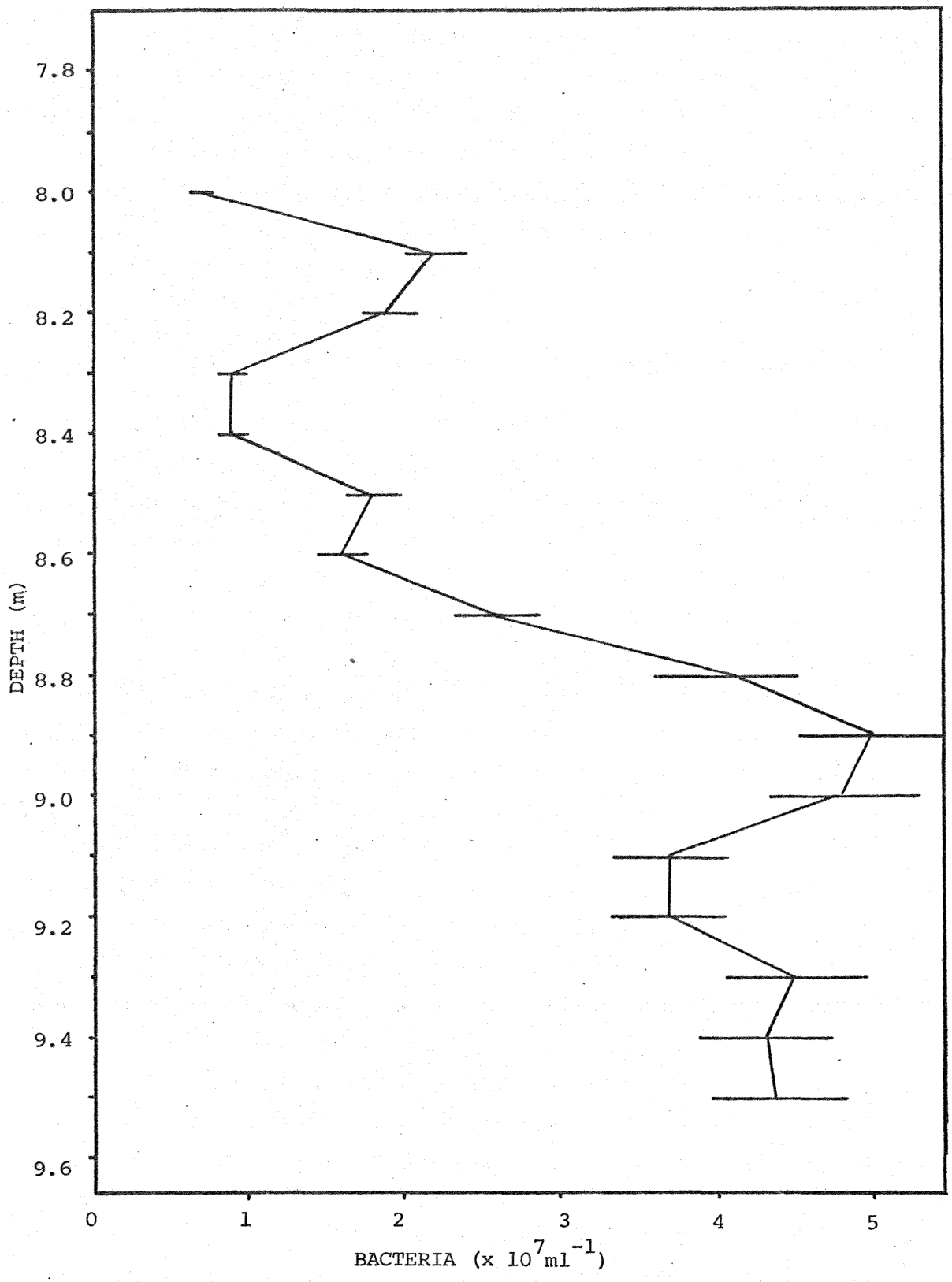


Figure 4.3/3 - The Distribution of Bacteria between 8.0m and 9.5m Lake Fryxell

Bacterial direct counts by epifluorescent microscopy

Bars indicate 95% confidence limits based on a sample size of 400

Over the sampling period broadening of the 9m peak, both upward and downward, occurred. Although part of this effect was undoubtedly due to the early lability of extracts, coupled with the way that the results are expressed, the changing shape of the major peak with time suggests that upward and downward spread of the photosynthetic bacteria did occur. Some groups of the Rhodospirillales (photosynthetic bacteria) are motile (Pfennig and Trüper, 1973).

4.3.2.4 ETS

This technique was, apparently, very close to its limits of sensitivity under the conditions in which it was employed. Differences in the variability of blank values account for the wide range of the calculated limits of detection.

Presumably the ETS peaks at about 8.2m and 8.4-8.5m (see figure 4.3/2) correspond to peaks of algal activity as algal biomass at these depths far exceeds bacterial biomass. However, it is noticeable that on three out of five dates the 8.4m peak is displaced downward in relationship to the corresponding chlorophyll peak. Possibly this is due to the presence of more than one type of alga at this depth.

Broadening of the ETS peak occurs for both mid-day samples and at 2400h on 30/12/80 the dramatic reduction in the 8.4-8.5m peak height corresponds to the upward migration of the corresponding chlorophyll peak. That the ETS peak, on this occasion, remains constant at 8.5m is further evidence for a heterogeneous algal population, only part of which migrates. Alternatively, the remaining ETS activity may be associated with the peak in bacterial numbers which was subsequently shown to occur at this depth on 8/1/81 (see figure 4.3/3).

The high ETS activity above 8.2m at 1300h on 28/12/80 may be due to the downward migration of an algal population from above 7.8m. This peak does not coincide with a similar one on the corresponding chlorophyll profile.

By virtue of a low variability in blank values, the calculated limit of detection of the ETS technique (see section 2.5) is low enough for the results of 0100 hours on 2/1/81 to be significant below 8.6m. The resulting profile is similar in shape to the corresponding bacterio-

chlorophyll profile but is displaced downward by 20cm. Apart from on this one occasion, the ETS technique did not prove to be sufficiently sensitive for its intended purpose, which was to locate and observe any movement of peaks of bacterial activity below the chlorophyll peaks.

4.3.3 Summary of Preliminary Findings

As explained above, the purpose of the preliminary work was to assist in the location of a suitable depth at which to study diurnal changes in heterotrophic uptake kinetics. The results obtained, particularly the limited value of the ETS results, did not make this choice easy.

The chosen depth was 9m, largely because, up until 2/1/81 this coincided with a stable peak of bacteria and measurable uptake of both glucose and glycolate was known to occur at this depth. It was realised that glycolate extracellular release is not a phenomenon which has been associated with photosynthetic bacteria, the primary producers at this depth, and also that the stability of stratification would possibly flatten out any diurnal peaks in glycolate supply from the algae at 8.4m depth. Weighed against this was the effect that algal migration between 8m and 8.5m would have had upon a diurnal study of uptake kinetics. Changes in uptake kinetics might have reflected the changing population of algae, if these had exercised even a limited uptake capability, rather than the adaption of the associated bacteria to a changing supply of substrate. In retrospect, 8.6m might have been sufficiently close to the algal peak to receive released substrate, and yet sufficiently far removed so that the direct effects of migration on uptake kinetics could be ignored. However, the presence of sufficient bacteria to achieve significant substrate uptake at this depth, could not be determined using the techniques available. The results of the bacterial direct counts by epifluorescent microscopy (see figure 4.3/3) were not available until after my return to New Zealand.

4.4 DIURNAL STUDIES OF HETEROTROPHIC UPTAKE

4.4.1 Experimental Approach

The sampling regime involved a series of samples taken at either midnight or mid-day over a period of five days. It was not possible to measure heterotrophic uptake after each of the ten successive 12 hour intervals because of limitations in manpower but a total of seven samples were taken, three at midnight, two at mid-day and one in the evening (1545h). During the same five day period a sample was taken at midnight in order to determine the effect of incubation time on substrate uptake.

The reasons for the difference between this experimental approach and that used in Lake Rotomanuka (where uptake was to have been measured more times in each 24 hour period with each 24 hour period being separated by a longer time span) were as follows:

(a) It was hoped that by repeating diurnal samplings over a period of several days true diurnal (cyclic) changes would be distinguishable from other short term (non-cyclic) fluctuations in heterotrophic uptake.

(b) Because of the limited availability of incubation vessels and manpower, it was not possible to collect and incubate more than two samples in any 24 hour period.

(c) Because of the limited duration of the time available for experimental work at Lake Fryxell no attempt could be made to follow changes in heterotrophic uptake over the summer season.

Water samples were collected at each sampling time for subsequent direct counts of bacterial numbers.

Based on the preliminary findings on heterotrophic uptake (section 4.3.2.1) an incubation time of six hours was considered necessary to ensure detectable uptake. However, it was realised that an incubation time corresponding to such a large proportion of a 24 hour cycle might well render the techniques used insensitive to diurnal changes in uptake kinetics, which might occur during the course of the long incubation.

The results of an experiment in which a more careful appraisal of the effects of incubation time on substrate uptake was made, were not available until after all of the heterotrophic uptake experiments had been completed.

4.4.2 Results and Discussion

4.4.2.1 The Effect of Incubation Time on Glucose Uptake

Although the incorporation of ^{14}C-label into biomass appears to continue at a constant rate throughout the course of the experiments (see figure 4.4/1A), the rate of $^{14}CO_2$ production changes, increasing between 55 minutes and 120 minutes and decreasing thereafter. The effect of this rate change upon the percentage of ^{14}C respired is shown in figure 4.4/1B. These results indicate that the chosen incubation time of six hours was long enough to allow adaption of the sampled heterotrophic community to incubation conditions.

4.4.2.2 The Effect of Incubation Time on Glycolate Uptake

The glycolate results in figure 4.4/1A show an even more drastic effect of sample adaptation than the glucose results. Whether this adaptation was an active response, in which the organisms responsible for uptake modified their uptake systems to reduce the uptake of a substrate which they could not use or whether it was a passive response, in which glycolate caused damage or death to the cells concerned, cannot be ascertained. The effect was not caused by substrate depletion which did not exceed 5%.

4.4.2.3 The Effect of Substrate Concentration on Glucose Uptake

Examination of figure 4.4/2 reveals uptake kinetics which do not appear to obey the Michaelis-Menten equation. The only occasion on which a modified Lineweaver-Burke linearisation of the uptake data produced an acceptable straight line was at 0030h on 4/1/81 (figure 4.4/2B). The difference between this incubation and the others was that on this occasion the incubation was not performed in the dark. Illumination was the naturally occurring light level inside on an overcast morning (130 $\mu Em^{-2}s^{-1}$). Without knowing whether the uptake in the light is the result of a constant uptake rate over the incubation period, it is not possible to regard the measured kinetic parameters (V_{max}, ($K_T + S_n$) and T_t) which could be calculated from this set of data with any degree of confidence. This is even more true of results from dark incubations where the uptake rate was known to change during the incubation. However

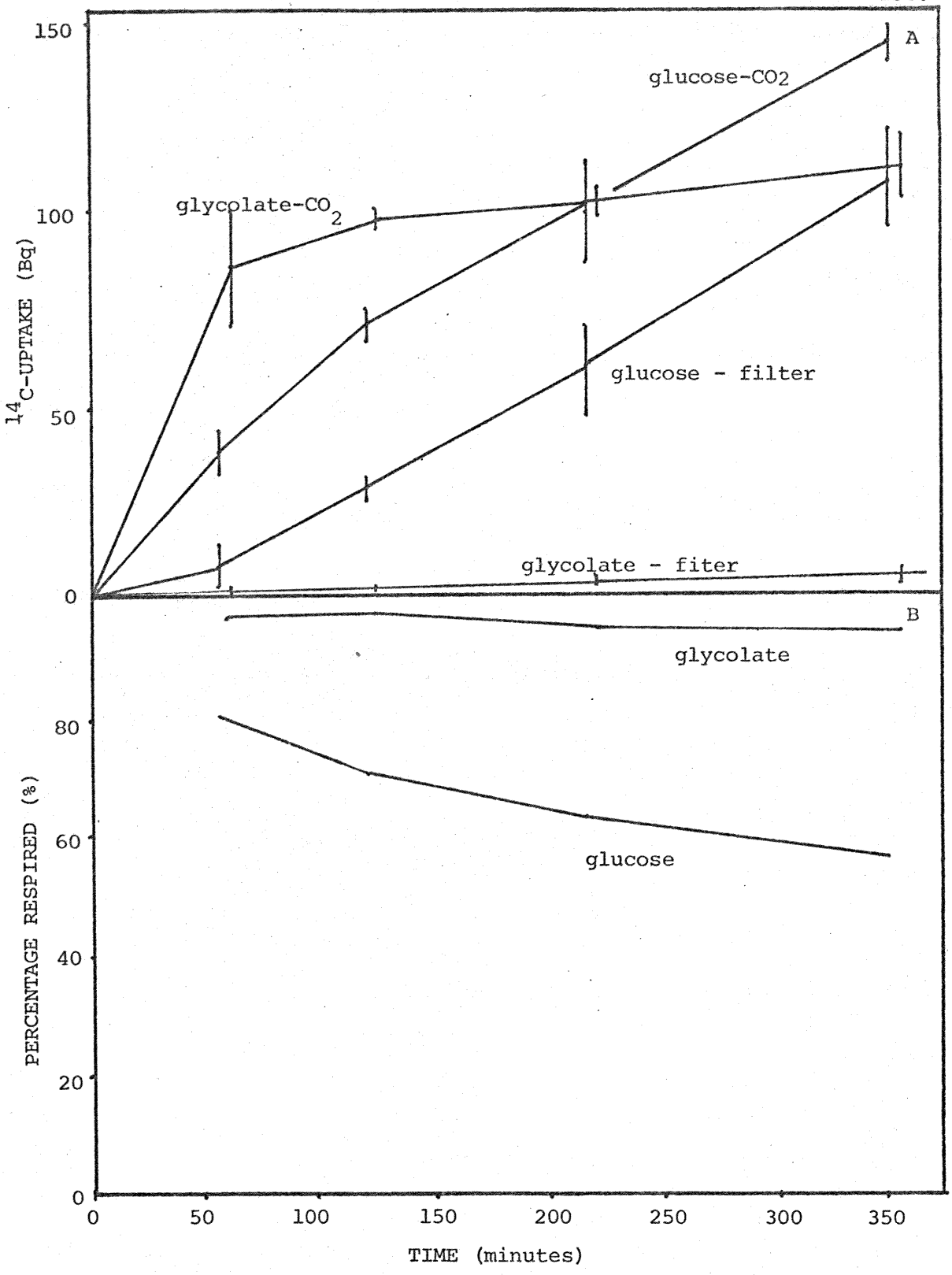


Figure 4.4/1 - The Effect of Incubation Time on Substrate Uptake and on the Partitioning of ^{14}C-label

Lake Fryxell 0045h 7/1/81

A - Substrate uptake

B - Fraction of substrate which was taken up and subsequently respired

Bars show the range of duplicate samples

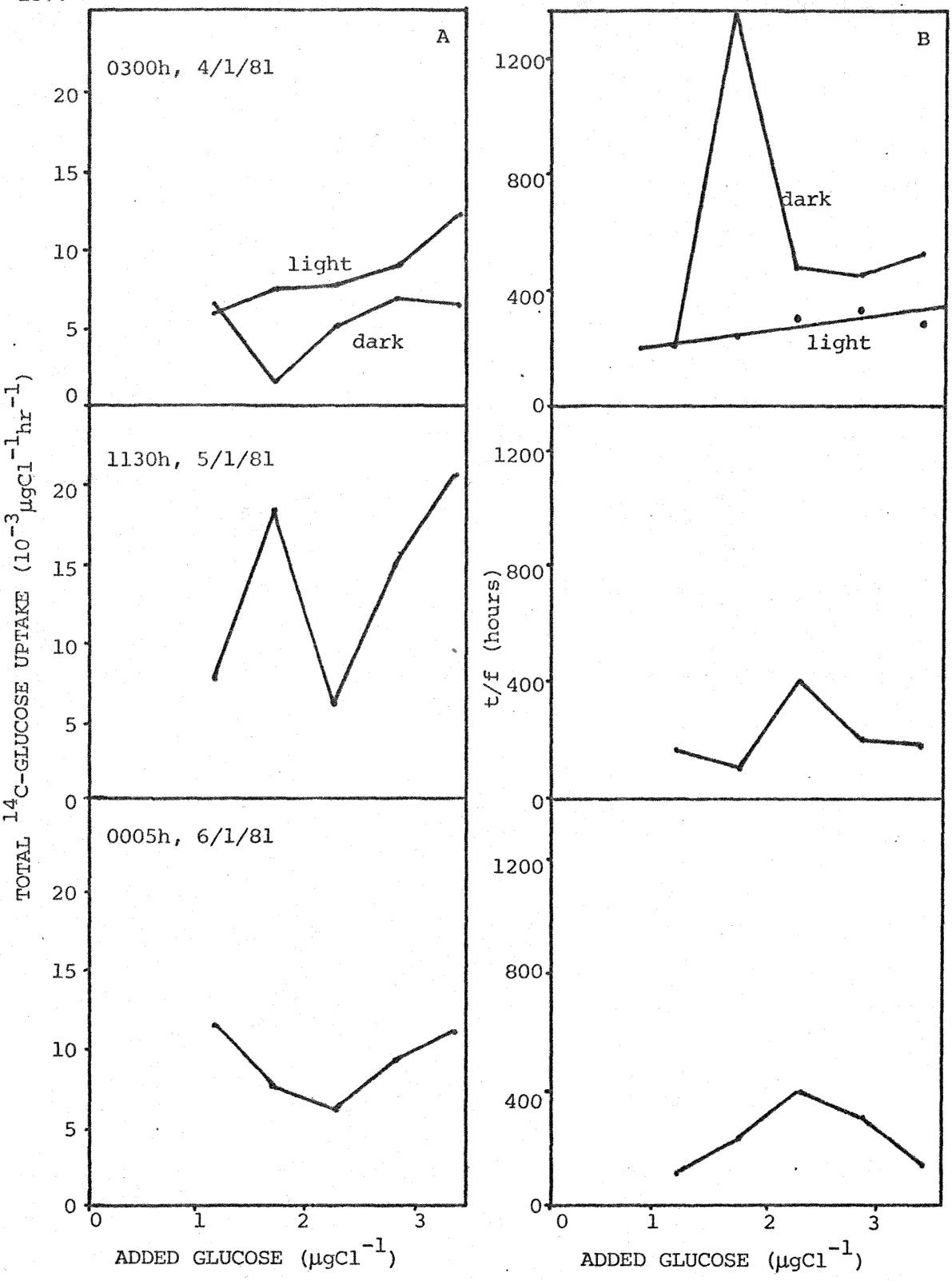


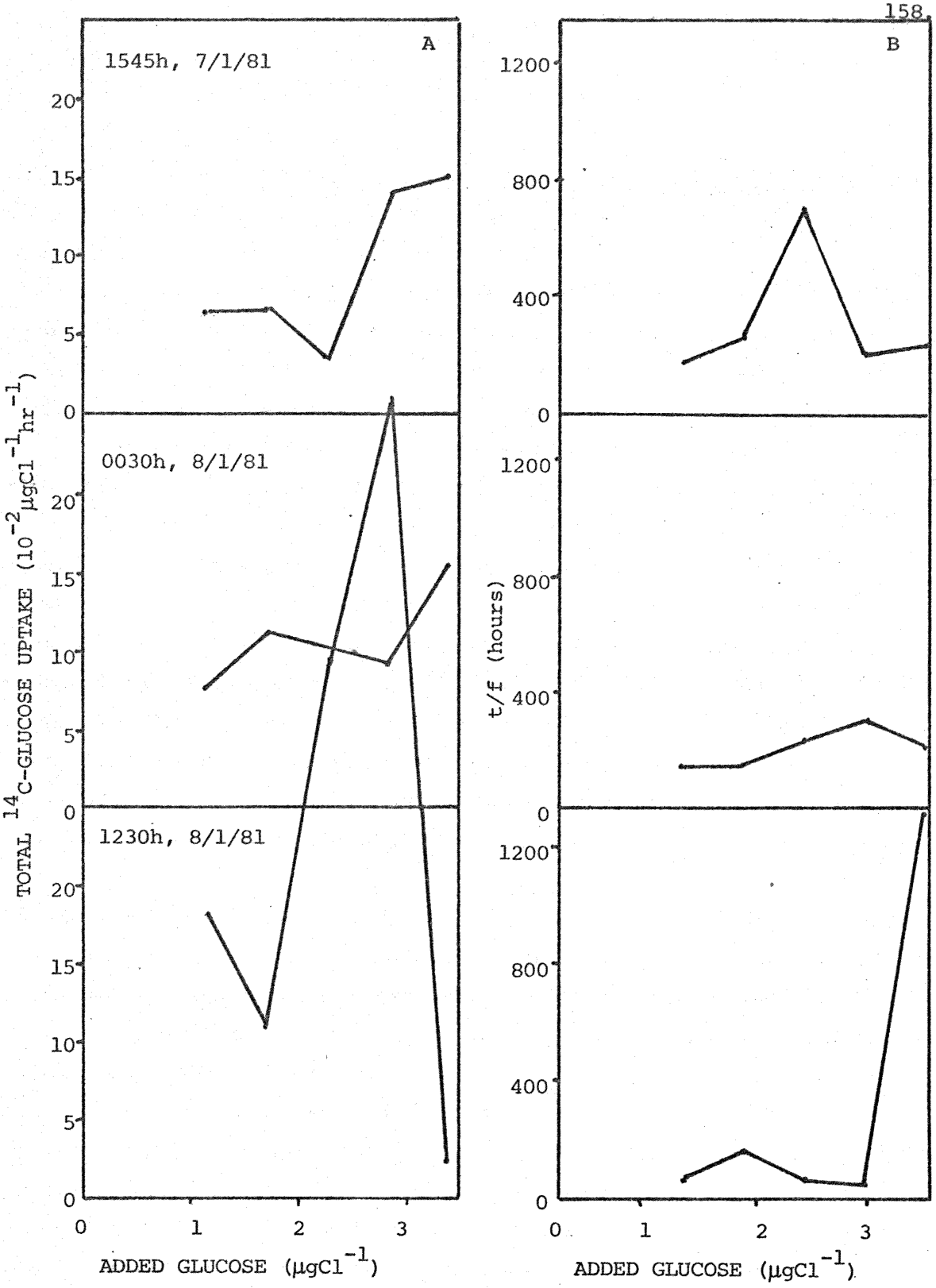
Figure 4.4/2 - The Effect of Substrate Concentration on Glucose Uptake - Lake Fryxell

A - Total uptake (incorporated ^{14}C plus $^{14}CO_2$) vs. Added glucose concentration

B - Modified Lineweaver-Burke plot

Incubation Temperature 3.2°C ± 0.5°C

Incubation Time 6hr


A
B
1545h, 7/1/81
0030h, 8/1/81
1230h, 8/1/81
TOTAL ^{14}C-GLUCOSE UPTAKE (10^{-2} µgCl^{-1}hr^{-1})
t/f (hours)
ADDED GLUCOSE (µgCl^{-1})
ADDED GLUCOSE (µgCl^{-1})

the mean uptake over all concentrations is greater in the light than in the dark, indicating that photoassimilation probably occurred.

Each of the dark incubations produced a plot of uptake against substrate concentration which has a negative slope over part of its range. Whilst this could be interpreted as experimental error in each case, the ranges of uptake results from duplicate incubations in figure 4.4/1 are small enough to suggest that this is not the case. This is also suggested by the fact that in each case, ^{14}C-respiration and ^{14}C-incorporation yield similarly shaped plots, although this is not shown in figure 4.4/2. Clumping of bacteria could be a cause for the apparently anomolous results (discussed in section 3.3.2). However, this also appears unlikely given the small range of results from duplicates in figure 4.4/1. No unique cause for these non-standard relationships between uptake and concentration is obvious. Contributing factors might be (i) variation of magnitude of induction of uptake systems in response to different concentrations of added substrate; (ii) inhibition of uptake with increasing concentration; (iii) parallel uptake systems with different kinetics; (iv) sigmoidal uptake kinetics (see section 3.2.4.3). A combination of these factors could, theoretically, produce the relationships observed in figure 4.4/2.

No diurnal trend is apparent in the results for glucose uptake. However, the percentage of ^{14}C respired to CO_2 after the substrate had been taken up, changed dramatically between sampling dates (figure 4.4/3B). The most likely explanation for this is the migration of different populations into and out of the sampled depth. An alternative explanation might be that modifications of the uptake systems within a stable population do occur on a diurnal basis, resulting in a decreased percentage respiration during the day, but that light levels immediately prior to mid-day on 8/1/81 were sufficiently low to induce the uptake systems normally found at night. This explanation is discredited by the fact that the morning of 8/1/81 was bright and clear with less than $^{2}/8$ cloud cover at most times.

The variation between bacterial numbers on the different sampling dates (see figure 4.4/4) could be attributed to migration although population growth and decline cannot be discounted.

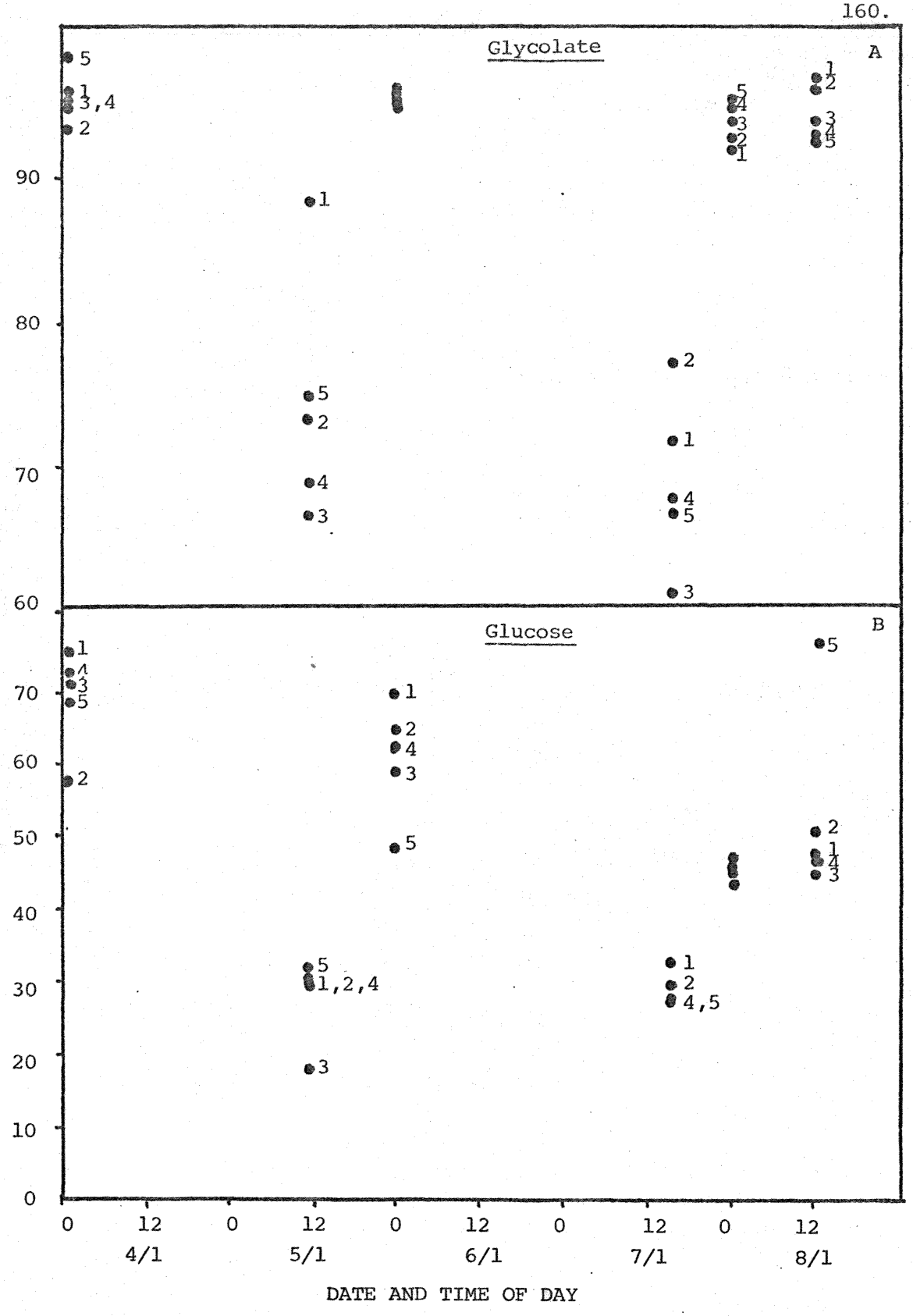


Figure 4.4/3 - Variation in Partitioning of ^{14}C-Activity between Sampling Times - Lake Fryxell

A - Glycolate

B - Glucose

Numbers refer to the concentration of added substrate in the incubation, ranging from 1, the lowest, to 5, the highest

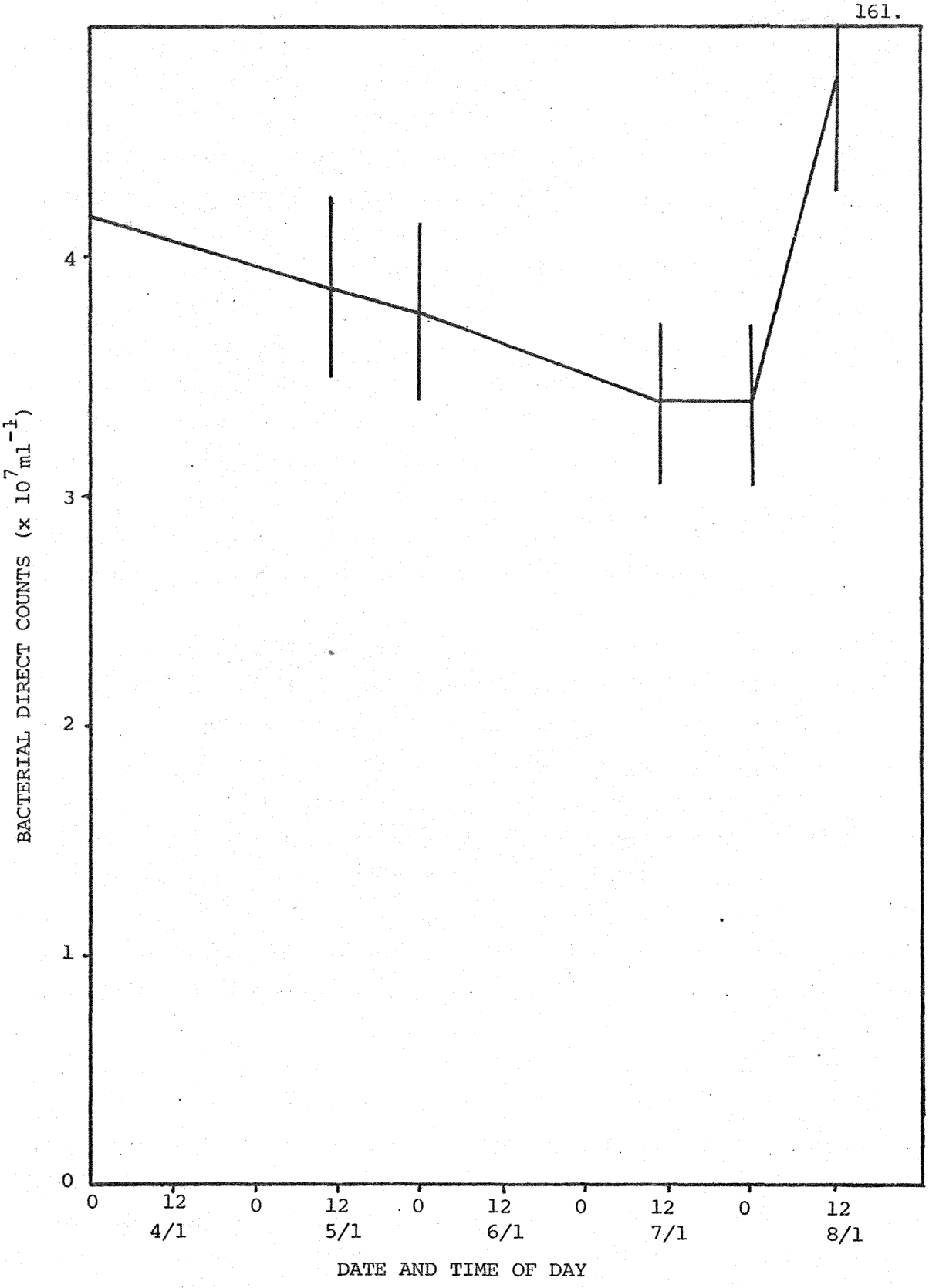


Figure 4.4/4 - Variation of Bacterial Numbers between Sampling Times - Lake Fryxell

Bars indicate 95% confidence limits based on a sample size of n=400

4.4.2.4 The Effect of Substrate Concentration on Glycolate Uptake

On three occasions it was possible to fit straight lines to the modified Lineweaver-Burke linearisations of the uptake data and achieve regression coefficients greater than 0.75 (see figure 4.4/5B, 0030h: 4/1/81, light and dark and 1130h: 5/1/81). However, as the relationship between glycolate uptake and incubation time has been shown to be non-linear, the kinetic parameters which could be obtained from these plots would be meaningless. This example highlights the warnings of Jannasch (1974) and Vaccaro and Jannasch (1967) that linearity of the modified Lineweaver-Burke relationship does not necessarily imply the presence of Michaelis-Menten kinetics.

As with glucose uptake, glycolate uptake was greater in the light, indicating that photoassimilation was probably occurring.

Another similarity with the glucose results is the decrease in the fraction of substrate which was taken up and subsequently respired at 1130h: 5/1/81 and 1545h: 7/1/81 (see figure 4.4/3A). This similarity suggests that the variation experienced in the glucose results was not the result of some experimental artifact such as a variation in the level of $^{14}CO_2$ present in separate bijou containers of substrate, in which case migration of different populations into and out of the sampled depth still seems to be the most probable cause for the differences in the partitioning of ^{14}C-activity between biomass and respiration on different dates.

There is an apparent trend relating the mean uptake over all added concentrations and the time of day of sampling. This trend is one of decreased uptake in the two samples taken at mid-day and the evening sample compared with the three midnight samples. However, the value of this observation is limited, in the context of the aims of this thesis, if, as has just been suggested, migration is occurring about the 9m sampling depth.

The actual relationships observed between glycolate uptake and glycolate concentration vary.

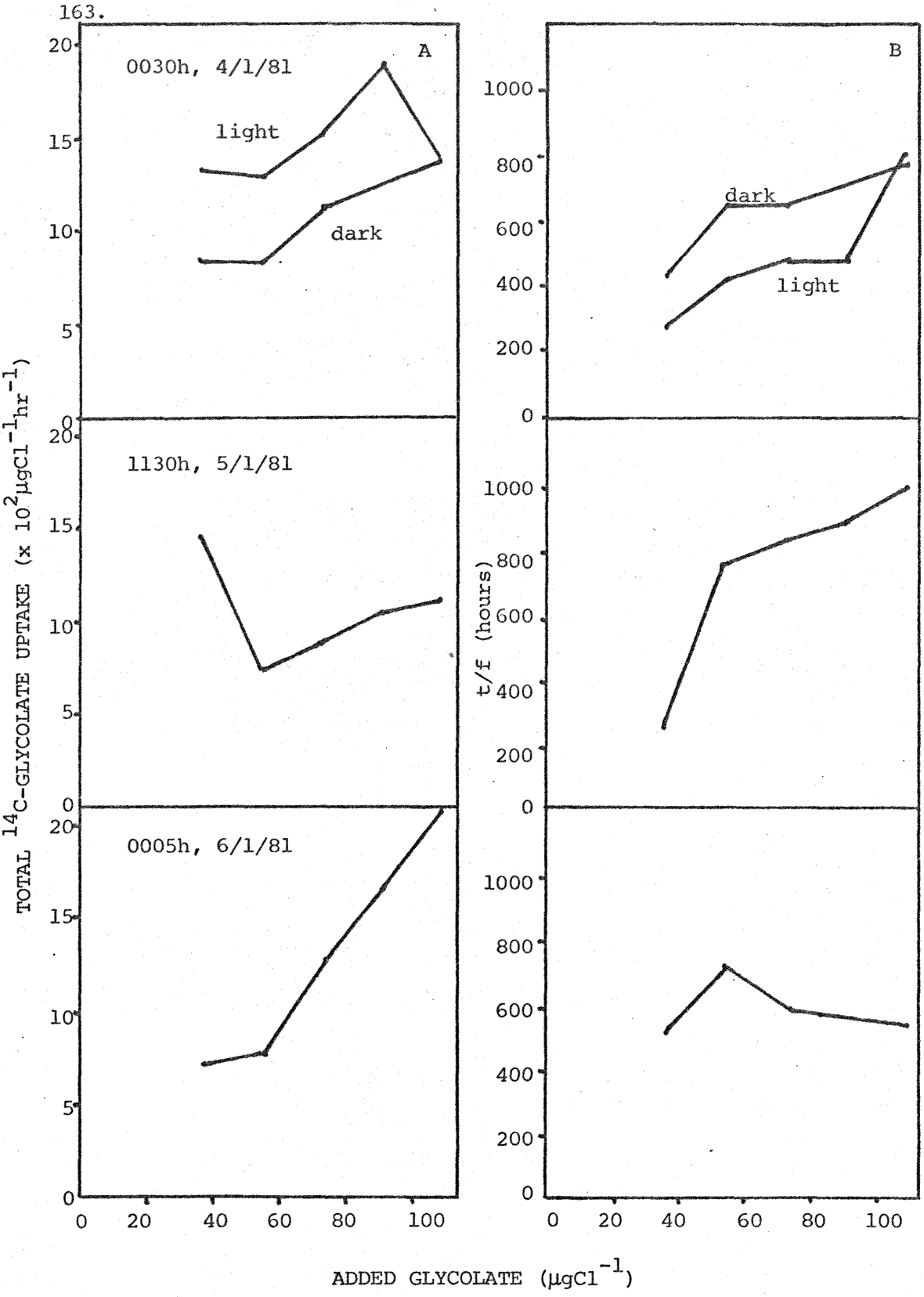


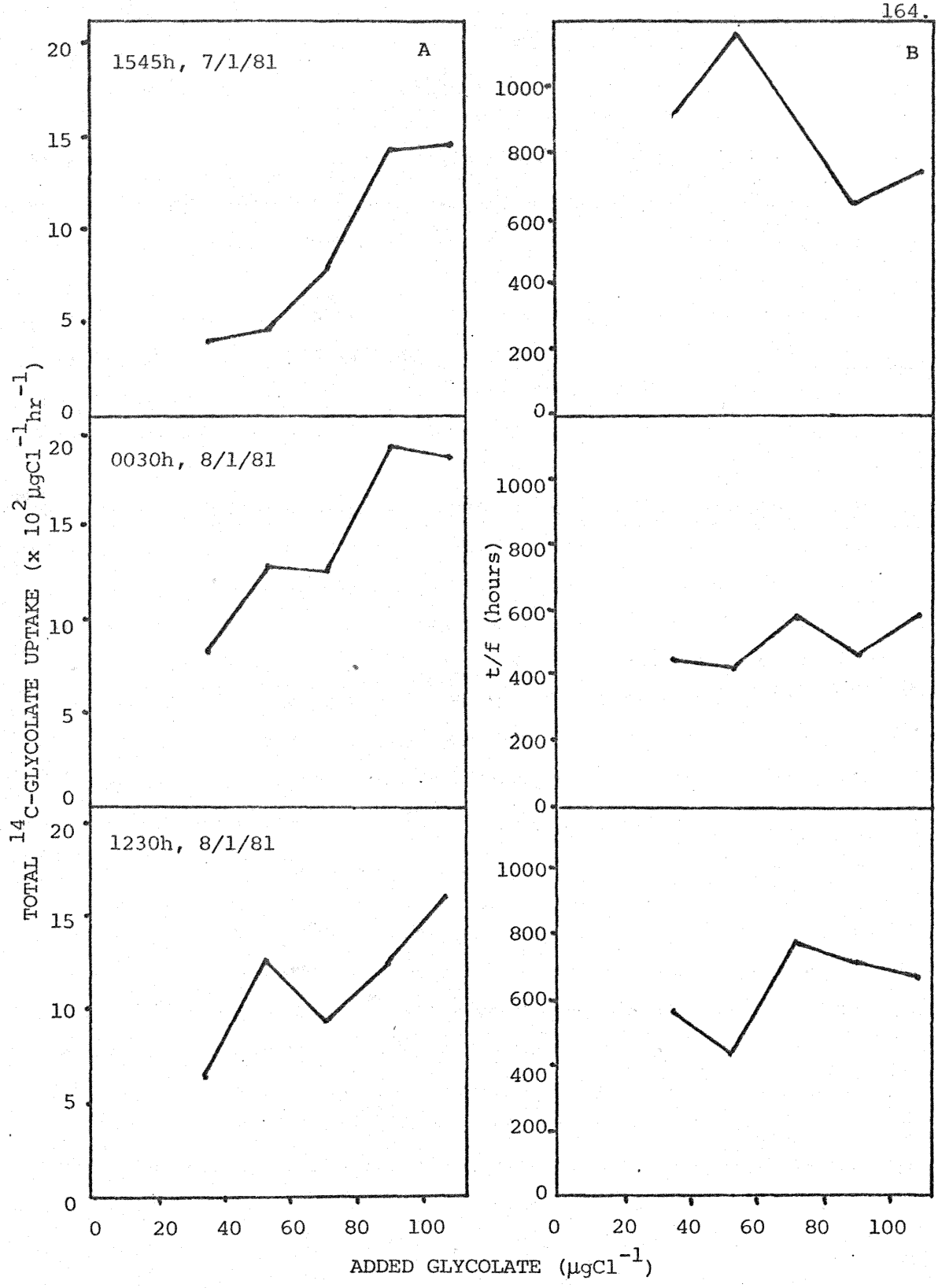
Figure 4.4/5 - The Effect of Substrate Concentration on Glycolate Uptake - Lake Fryxell

A - Total uptake (incorporated ^{14}C plus $^{14}CO_2$) vs. Added glycolate concentration

B - Modified Lineweaver-Burke plot

Incubation Temperature 3.2°C ± 0.5°C

Incubation Time 6hr


A
B
1545h, 7/1/81
0030h, 8/1/81
1230h, 8/1/81
TOTAL ^{14}C-GLYCOLATE UPTAKE (x 10^2 µgCl^{-1}hr^{-1})
t/f (hours)
ADDED GLYCOLATE (µgCl^{-1})
0
5
10
15
20
200
400
600
800
1000
0
20
40
60
80
100

4.5 NATURAL GLYCOLATE CONCENTRATION

At each sampling time samples of lake water were absorbed on to alumina and stored for subsequent glycolate analysis (see section 2.6 for a discussion of the technique and figure 4.5/1 for the results).

As was mentioned in section 2.6 the added glycolate standards failed to produce the expected colour development when extracted from the alumina and assayed with 2,7-dihydroxynaphthalene. Whether this was due to loss of glycolate from standards before or after absorption on to the alumina or to a lower efficiency of extraction than was usual of glycolate from the alumina, cannot be determined and has a bearing on the interpretation of the results. If the loss occurred prior to absorption (but not after), use of the slope obtained when the technique was used in distilled water with the intercepts obtained from each set of Fryxell results produces the lower limit of the range shown in figure 4.5/1B. Assuming that loss occurred during storage of the alumina or that extraction of glycolate from the alumina was far less efficient than normal would necessitate the use of the Fryxell results as obtained and would result in the upper limit of the range in figure 4.5/1B.

Using either assumption produces the result that (i) glycolate concentration did not change on a diurnal basis, and (ii) glycolate concentration decreased by 24% or 38% (depending on which set of results from figure 4.5/1B are used) over the sampling period. Although the former result (i.e. (i) above) is likely to be due to a lack of change in both glycolate supply and glycolate demand on a diurnal basis, it must be borne in mind that simultaneous changes in both supply and demand can give rise to a constant concentration. The latter result (i.e. (ii) above) is presumably due to some seasonal trend.

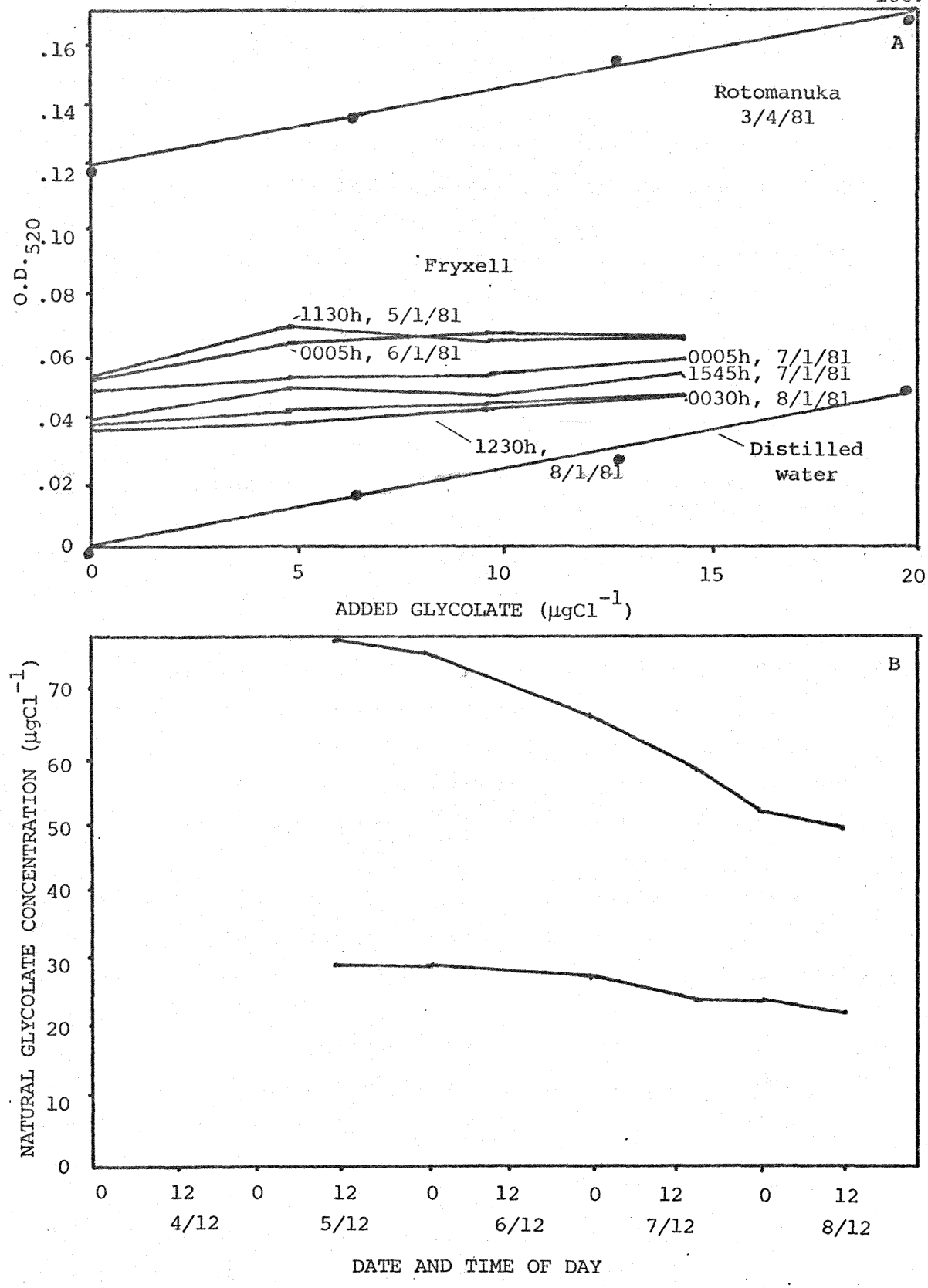


Figure 4.5/1 - Glycolate Concentration - Lake Fryxell

A - Variation in colour development with concentration of standard

B - Calculated glycolate concentrations at different sampling times

Upper limit calculated from mean slope of all Fryxell data in 4.5/1A with mean O.D. for each sampling time

Lower limit calculated from slope from Rotomanuka plot in 4.5/1A with x-intercept of each set of Fryxell data

See text for explanation (sections 2.6 and 4.5)

4.6 GENERAL DISCUSSION AND SUMMARY

As far as the aims of this thesis are concerned, the results obtained in Lake Fryxell are equivocal. That is, they do not provide evidence either for or against the hypothesis that heterotrophic populations in such lakes modify their uptake systems as a response to changing substrate supply on a diurnal basis. There are two major factors contributing to the equivocal nature of the conclusions.

(i) It was not possible to determine valid heterotrophic uptake kinetic parameters because adaption of uptake systems occurred during incubation and because attempts to linearise the relationship between substrate uptake and substrate concentration, using the modified Lineweaver-Burke technique, were not always successful.

(ii) There was evidence that migration of organisms into and out of the sampled depth, was occurring. This evidence consisted of changes in bacterial numbers at the sampled depth and changes in the partitioning of ^{14}C-activity between biomass and respiration, neither of these changes being diurnally cyclic.

A positive result was the finding that glycolate concentration did not vary on a diurnal basis, suggesting that changes in glycolate concentration on a diurnal basis could not provide a stimulus for changes in the uptake kinetics for glycolate.

Other positive aspects of the work which are not related to the main aims of the thesis are:

(i) Further evidence for diurnal migration of algae within the photic zone;

(ii) The discovery of a photosynthetic bacteria at the oxycline;

(iii) The observation that the microstratification of biological activity in Lake Fryxell is dynamic on both a day-to-day basis and a seasonal basis.

Chapter 5

A RETROSPECTIVE CRITIQUE OF THE METHODOLOGICAL APPROACH USED IN THIS THESIS

5.0 A RETROSPECTIVE CRITIQUE OF THE METHODOLOGICAL APPROACH USED IN THIS THESIS

In both of the lakes in which studies were made, differences were observed in the uptake of ^{14}C-labelled substrates by aquatic micro-organisms from water samples which were taken at different times of day. In Lake Rotomanuka the differences were seen as differences in the relationship between substrate uptake and concentration of added substrate, i.e. as differences in substrate uptake kinetics, and some of the observed changes in uptake kinetics were quantifiable in terms of significant differences between the kinetic parameters, V_{max}, T_t and $(K_T + S_n)$, when these parameters were measured at different times of day. In Lake Fryxell, the observed differences in substrate uptake were most apparent as differences in the partitioning of ^{14}C-labelled substrate between biosynthetic pathways and respiration to $^{14}CO_2$.

However, it was not possible to draw clear conclusions from these observed differences with respect to the major aim of this thesis, which was to examine the possibility that diurnal changes in the extracellular release of certain substrates by algae might result in a depletion of these substrates at night, which in turn might provide a stimulus for heterotrophic micro-organisms to adjust their capacity to take up the substrates in question.

In hindsight, this lack of firm conclusions was due to two inadequacies in the methodological approaches to the above-mentioned studies. These were:

(a) Adequate controls were not set up to determine whether the same heterotrophic population was being studied at each time of day. That is, it was not possible to determine whether migration of different populations into and out of the sampling site or alternatively, heterogeneous distribution of heterotrophic organisms within the studied water bodies, resulted in the observed differences in substrate uptake. The adopted approach to this problem did not adequately exclude the possibility of the occurrence of migration or heterogeneous population distribution. The approach used here was to observe the sampled population using a variety of techniques, including microscopic direct counts, chlorophyll extraction and measurement of INT dye

reduction. In retrospect, a more suitable approach might have involved the measurement of heterotrophic uptake kinetics at a variety of depths at several sampling sites at each time of day. An observed similarity between changes in substrate uptake kinetics, measured over a 24 hour period at the same depth but at different sampling sites, might be taken as evidence that the observed changes were not due to random sampling from a heterogeneous population at each time of day. Alternatively, an observation that patterns of change of uptake kinetics were different at different, but closely adjacent, sampling sites might be taken as evidence that a heterogeneous distribution of heterotrophs in the horizontal plane was the cause of observed changes in uptake kinetics.

Similarly, the effects on uptake kinetics of vertical migration might be better understood by observing changes in uptake kinetics at a variety of depths. To achieve this in Lake Fryxell, because of the fineness of stratification, it would be necessary to sample at a large number of discrete depths, or alternatively, to use a sampling device which would take a smaller number of integrated samples, each from a given range of the depth profile.

(b) The second failing of the adopted methodological approach was that inadequate attention was paid to the possibility that observed changes in uptake kinetics were not diurnally cyclic. In Lake Rotomanuka, on the one occasion when uptake kinetics were measured over a complete 24 hour period, different uptake kinetics were observed at the same time of day on two adjacent days. However, that does not exclude the possibility that at least part of the pattern of change of uptake kinetics, at that time of year, was diurnally cyclic. In this respect, the methodological approach which was adopted at Lake Fryxell in which uptake kinetics were measured at two different times of the day over a period of five days, was a superior approach. Measuring uptake kinetics at regular intervals of several hours over a period of several days would have provided more information on the periodicity, if any, of short term changes in uptake kinetics.

Each of the improvements to methodological approach which are suggested above, involve the measurement of uptake kinetics in a much larger number of samples at narrower time intervals than could be considered in this study, for reasons of resources and manpower. However, it might be feasible, with similar resources and manpower, to undertake a study which concentrated on the measurement of one kinetic parameter only. Because changes in both T_t (turnover time) and $(K_T + S_n)$ (the sum of the uptake rate constant and the natural substrate concentration) are influenced by changes in S_n (the natural substrate concentration), the most valuable parameter to consider in isolation is V_{max} (the theoretical maximum attainable uptake rate). (See section 2.1(a)). It is possible to measure an uptake rate which approximates to the value of V_{max} by measuring uptake at a single concentration of added substrate which is chosen so as to be high enough to saturate high affinity uptake systems and to mask the effects of changes in natural substrate concentrations but which is not so high that any measured uptake is that resulting from low affinity uptake systems (which are relatively unimportant with respect to uptake at the low concentrations of naturally occurring substrate). Griffiths *et al* (1977) found a high correlation ($r = 0.90 - 0.98$ in sediment and $0.77 - 0.95$ in water) between the V_{max} of glutamate uptake, estimated using the kinetic approach of Parsons and Strickland (1961), and uptake of glutamate at a single saturating concentration of added glutamate. Provided that a suitable substrate concentration was chosen with respect to the factors discussed above and also with respect to the apparent inhibition of substrate uptake at high concentrations of added substrate which occurred in this study, then measurement of substrate uptake at a single concentration of added substrate, but with greater frequency and at a greater number of sampling sites and depths than was possible in this study, might be a very useful methodological approach for the study of regular diurnal changes in the induction of substrate uptake systems by aquatic heterotrophs.

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