

A STUDY OF NITRIFICATION IN LAKES OF THE ENGLISH LAKE DISTRICT

A thesis submitted for the degree of Doctor of Philosophy

by

Grahame H. Hall

Department of Biochemistry, Brunel University

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## Abstract

Nitrification became the dominant nitrogen transformation in a number of lakes which accumulated ammonium, in hypolimnetic water, under aerobic conditions. The timing and duration of this activity varied between lakes but was characterized by decreasing ammonium, and increasing nitrate, concentrations. In Grasmere lake this phase was found to be due to the activity of planktonic chemolithotrophic nitrifying bacteria. The observed nitrate concentration increased during this phase and accounted for up to 15% of the total oxygen deficit of the hypolimnion. At similar in situ temperatures nitrification rates and nitrifying bacterial populations were greater in oxidised sediments than in the water column. Littoral sediments were more important than profundal sediments as sites of nitrification due to higher temperatures and the persistence of oxidising conditions to greater depths into these deposits. Within the littoral zone the physical characteristics of the sediment were important with organic rich deposits sustaining greater rates of nitrification than sandy sediments. Nitrate reduction was the primary determining factor of nitrate concentrations in sediment interstitial waters. Some observations on the culture of chemolithotrophic nitrifying bacteria from lakewater were discussed.

Reviews of the methodology for nitrification research and nitrification in the lacustrine environment are presented.

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## List of Abbreviations

1. Phase I organisms : Ammonia oxidising lithotrophic bacteria
2. Phase II organisms: Nitrite oxidising lithotrophic bacteria
3. F.A. : Fluorescent antibody
4. CO<sub>2</sub> uptake : Dark carbon dioxide uptake
5. Throughout this thesis ammonia in aqueous solution is referred to as ammonia although it is realised that at the pH value encountered (6.0 to 7.6) very little would be present in the undissociated form
6. Organisms which obtain energy and/or cellular carbon from organic substrates have been referred to as heterotrophs. This was to avoid the cumbersome term chemoorganotroph.

## CHAPTER 1

## Introduction

## 1.1 General

Nitrogen exists in the biosphere in a number of forms, the major ones being nitrate, nitrite, ammonia, organic nitrogen and nitrogen gas. These various forms of nitrogen are inter-related by a series of reactions which collectively constitute the nitrogen cycle. This cycle represents the flow of nitrogen between particulate living systems and soluble inorganic forms. Figure 1.1 (from Wetzel, 1975) is a diagram of the nitrogen cycle as it may occur in an idealized stratified lake. Similar diagrams have been presented by Brezonik (1972) and Kuznetsov (1970) but this representation is preferred because it illustrates some of the interactions between the nitrogen and the carbon cycles thus yielding a better appreciation of the complexity of the ecosystem. Figure 1.1 demonstrates that the nitrogen cycle in the lacustrine environment is, with the exception of exchange reactions (No. 7), totally biochemical in nature. However, in eutrophic lakes deoxygenation of the hypolimnion, and subsequent reduction of profundal sediments, can greatly increase the rate of ammonia exchange from sediments to the water column.

Nitrogen transformations in the nitrogen cycle involve a series of oxidative and reductive reactions. In natural systems the nitrogen atom can exist in oxidation states ranging from +5 ( $\text{HNO}_3$ ) to -3 ( $\text{NH}_4^+$ ) (Aleem, 1970). Most biochemical redox reactions occur in discrete stages involving the transfer of two electrons and therefore it is not surprising that nitrogen compounds of various intermediate oxidation states have been implicated in the inorganic nitrogen metabolism of either intact organisms or their cell free preparations (Nason, 1962).

It is also apparent in Figure 1.1 that ammonification, nitrification and assimilation reactions represent an internal cycling of combined nitrogen

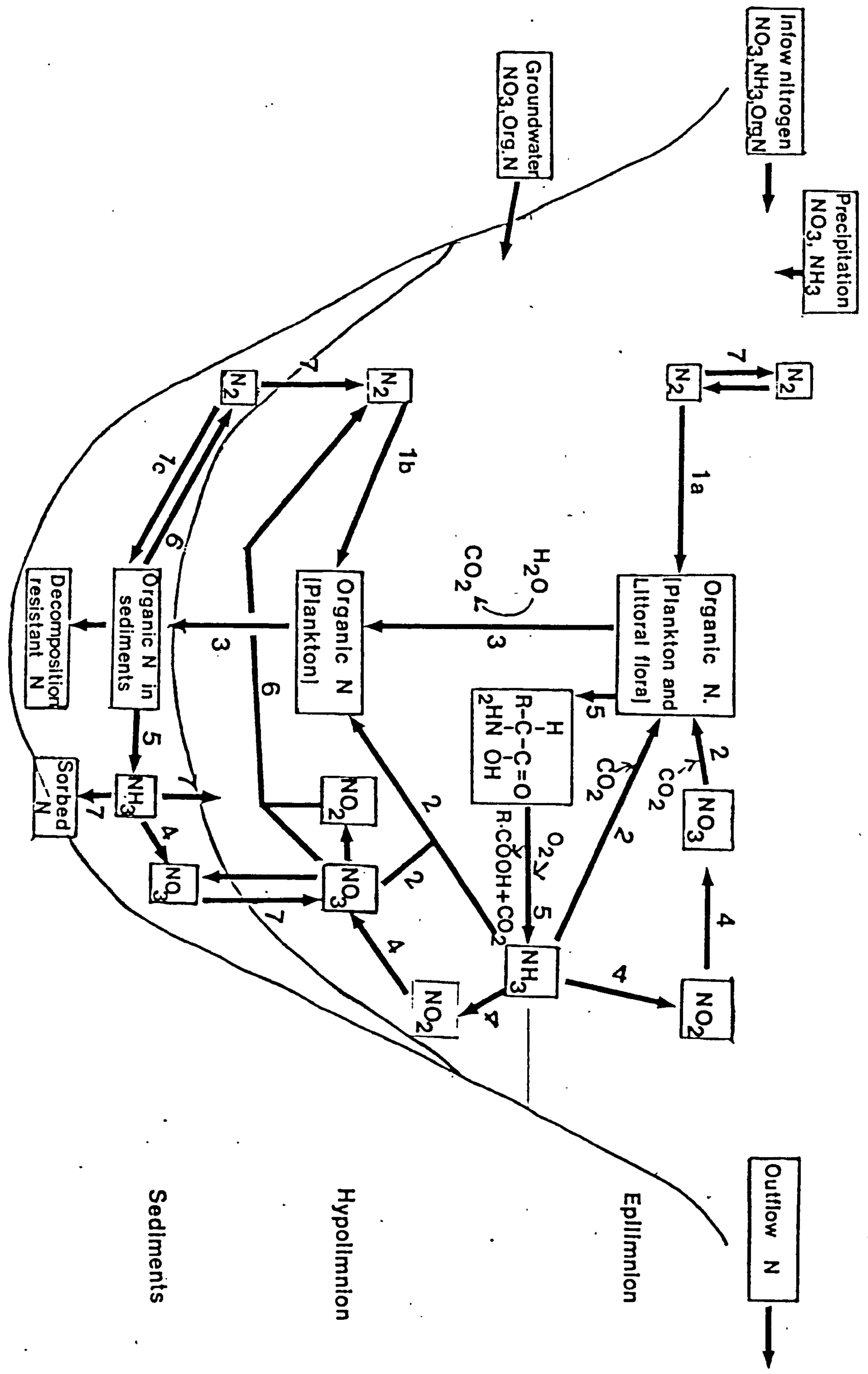


PTO

FIGURE 1.1 Schematized representation of the nitrogen cycle in an idealized stratified lake.

Key to notation and definitions of reactions.

1. Nitrogen fixation: The reduction of molecular nitrogen ( $N_2$ ) to ammonia which can be incorporated into nitrogenous components of the cell.
  - 1.A. Aerobic nitrogen fixation mediated by certain blue-green algae eg. Anabaena and bacteria eg. Azotobacter.
  - 1.B. Aerobic nitrogen fixation in the absence of light mediated by bacteria eg. Azotobacter.
  - 1.C. Anaerobic nitrogen fixation mediated by bacteria eg. Clostridium and also by some photosynthetic bacteria eg. Chlorobium.
2. Nitrogen assimilation: The assimilation of inorganic nitrogen compounds into cellular material. Organic nitrogen can also be assimilated, this reaction is not shown in Fig. 1.1.
3. Sedimentation and Decomposition: Sedimentation is self explanatory. Decomposition refers to the catabolism (both aerobic and anaerobic) of organic carbon compounds.
4. Nitrification: The conversion of inorganic nitrogen compounds from a reduced to a more oxidised state. Nitrification of organic nitrogen can also occur, this reaction is not shown in Fig. 1.1.
5. Ammonification: The release of ammonia during the decomposition of nitrogen containing organic compounds. The release of amino acids is also included in this definition.
6. Denitrification: The reduction of  $NO_3$  to  $N_2$  via a series of intermediates which involves a terminal electron transport system.
7. Exchange reactions: Non-biological reactions occurring by diffusion along concentration gradients.



species within the ecosystem. On the other hand nitrogen fixation and denitrification represent nitrogen sources into and nitrogen sinks from the ecosystem respectively.

Aerobic and anaerobic nitrogen transformations should not be regarded as being totally mutually exclusive as (a) aerobic environments can sustain anaerobic microniches (Jorgenson, 1977), (b) concurrent nitrification - denitrification has been demonstrated at aerobic and anaerobic interfaces and (c) Meiberg et al (1980) have obtained evidence, in culture, for aerobic denitrification ( $[O_2] < 2.3 \text{ mg l}^{-1}$ ), the ecological significance of this reaction is, as yet, unknown.

Reviews devoted to aspects of the nitrogen cycle other than nitrification are provided by Brezonik (1968), Painter (1970, 1977) for descriptions of all nitrogen cycle reactions; Postgate (1978) and a collection of papers in Ecological Bulletins NFR 26 for nitrogen fixation and Dalton (1979) for the metabolism of  $NH_4^+$ ,  $N_2$  and  $NO_3^-$  by microorganisms.

## 1.2 Nitrification

Classically nitrification has been regarded as the oxidation of ammonia to nitrate, via a nitrite intermediate, solely carried out by chemolithotrophic bacteria. However, this definition has now been revised to 'the conversion of nitrogen compounds from a reduced to a more oxidised state', to include all the reactions known to be carried out by a wide range of microorganisms. The basic classifications of these organisms and the reactions they are known to mediate is shown in Figure 1.2.

Figure 1.2 Definition of nitrification and organisms known to mediate the process

DEFINITION

Conversion of nitrogen compounds from a reduced to a more oxidised state.

ORGANISMS INVOLVED

Group 1.

Chemolithotrophic bacteria

Two physiologically distinct groups

A. Oxidising AMMONIA to NITRITE

B. Oxidising NITRITE to NITRATE

Group 2.

Heterotrophs

Two morphologically distinct groups.

A. BACTERIA

B. FUNGI

Substrates and products can be organic or inorganic nitrogen containing compounds. The nitrogen atom in the product is always at a higher oxidation state than in the substrate.

### 1.3 Organisms known to mediate nitrification reactions

In this discussion the data available on the occurrence of these processes in the lacustrine environment will be omitted as they are reviewed in Chapter 3.

#### a) Chemolithotrophic nitrifiers

These are the classical nitrifying bacteria, isolates of which were first obtained, in pure culture, by Winogradsky (Winogradsky 1890). It is interesting to note that the classification of these organisms in the 7th edition of Bergeys Manual of Determinative Bacteriology (1957) was based mainly on the work of Winogradsky. This classification was revised by Watson (1974) and updated by Belser (1979) from which Table 1.1 is reproduced. Nitrobacter winogradskyi represents the grouping of organisms previously known as N. agilis and N. winogradskyi although isolates of these organisms have been shown to be immunologically distinct (Rennie and Schmidt, 1977a; Josserand and Cleyet-Marel, 1979). Two physiologically distinct groups of chemolithotrophic nitrifiers are recognised, those oxidising ammonia to nitrite, hereafter referred to as phase I organisms, and those oxidising nitrite to nitrate, hereafter referred to as phase II organisms. These inorganic substrates are used as electron donors for energy production and this is why these organisms are regarded as chemolithotrophs. Growth, and therefore energy production, has been demonstrated for Nitrobacter agilis (Smith and Hoare, 1968; Steinmuller and Bock, 1976; Kaltoff et al. 1979) and N. winogradskyi (Kaltoff et al., 1979) in culture with only organic substrates. Growth rates were much slower than under lithotrophic or mixotrophic conditions (generation times of 85 to 150 hrs. as opposed to 13 hrs). In common with most other studies on these nitrifying organisms information on the heterotrophic growth potential is restricted predominantly to the genera Nitrosomonas (Pan and Umbreit, 1972) and Nitrobacter (see above).

Table 1.1 Current classification of the Nitrobacteraceae (from Belser, 1979)

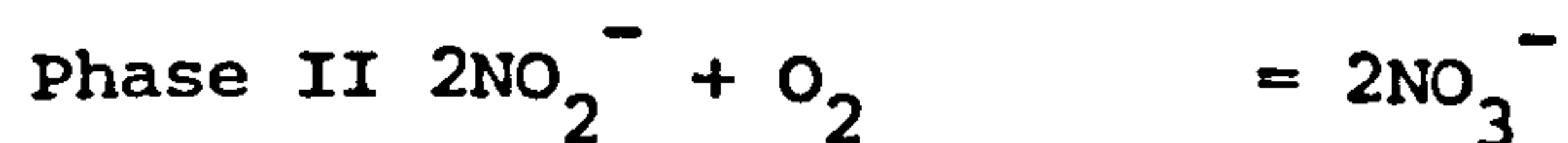
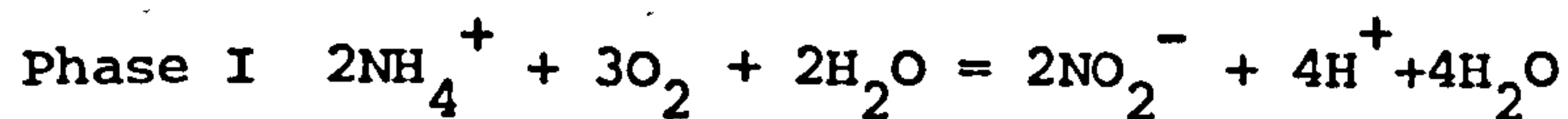
OXIDATION	GENUS	SPECIES	HABITAT
Ammonia to nitrite	Nitrosomonas	europaea	Soil, water, sewage
	Nitrospira	briensis	Soil
	Nitrosococcus	nitrosus	Soil
		oceanus	Marine
		mobilis	Marine
	Nitrosolobus	multiformis	Soil
	Nitrosovibrio	tenuis	Soil
Nitrite to nitrate	Nitrobacter	winogradskyi	Soil, water
	Nitrospina	gracilis	Marine
	Nitrococcus	mobilis	Marine

Matin (1978) states that the observations on Nitrosomonas have yet to be repeated and, moreover, heterotrophic growth of Nitrobacter may be related to differences between strains of these organisms. It is therefore difficult to classify these organisms as facultative or obligate chemolithotrophs. However, in the presence of their specific lithotrophic energy sources both Nitrobacter and Nitrosomonas isolates have been shown to assimilate various organic compounds (Steinmuller and Bock, 1976, 1977; Kaltoff et al, 1979; Delwiche and Finstein, 1965, Wallace and Nicholas, 1969). Under these mixotrophic growth conditions increased cell yields of Nitrobacter have been observed (Steinmuller and Bock, 1976; Garretson and San Clemente, 1978; Bock, 1976), as have increased activities for Nitrosomonas (Jones and Hood, 1980), in comparison to those obtained under autotrophic conditions. Moreover, Steinmuller and Bock (1976) noted a repression of CO<sub>2</sub> assimilating enzymes when Nitrobacter was grown under mixotrophic culture conditions. However, these observations do not exclude these organisms from the definition of autotrophs as organisms which are able to synthesise the carbon skeletons of their cell substances from inorganic carbonate (Schlegel, 1975). It is not yet clear whether, under the mixotrophic growth conditions which will be prevalent in the natural environment, they obtain the bulk of their biosynthetic carbon from carbon dioxide, thus conforming to the definition of autotrophs as proposed by Whittenbury and Kelly (1978).

Several reviews on nitrifying bacteria have appeared recently providing detailed descriptions of their nutrient requirements, biochemistry, growth kinetics etc. I will present a brief summary and discuss some of the most recent findings. General reviews on these organisms and the process of nitrification are provided by Walker (1975), Painter (1970, 1977), Focht and Verstraete (1977) and Sharma and Ahlert (1977). Nutrient requirements can be considered under the headings of macro and micro nutrition. The macro nutrients are regarded as the substrates (i.e.



inorganic nitrogen, oxygen and carbon source). Both phase I and II organisms are strict aerobes, the stoichiometrics of the individual reactions are as follows:



Stenstrom and Poduska (1980) have recently reviewed the effects of dissolved oxygen concentrations on the growth kinetics of these organisms and discuss the variability of the observations in terms of multiple substrate limited kinetics. Actual values for oxygen consumption have been found to be less than those predicted by the stoichiometry of the above equations (Wezernak and Gannon, 1968). The discrepancy cannot be explained by oxygen production during  $\text{CO}_2$  assimilation as proposed (see Wezernak and Gannon, 1968) as this oxygen is bound in organic compounds. Bhandari and Nicholas (1979), on the other hand, found exact oxygen stoichiometries for washed cell suspensions of Nitrosomonas europaea. Carbon requirements are given in the above reviews and in those references cited in relation to the definition of these organisms. The slow growth rates obtained for these organisms in culture are attributed, by some authors, to be a result of the failure to provide adequate growth conditions (Painter, 1970). A number of essential micronutrients have been identified, specifically for the genera Nitrosomonas and Nitrobacter, and these are reviewed by Painter, (1970, 1977); Wallace and Nicholas; (1969), Walker, (1975) and Sharma and Ahlert (1977). Many micronutrients are supplied in sufficient quantities as contaminants within the specific medium formulation, however, supplemental additions have often been demonstrated to be stimulatory (Painter, 1970). At high concentrations many micronutrients become toxic or inhibitory but as the threshold concentration varies with each element it is difficult to generalise on

this aspect. The ability of organic growth factors (e.g. biotin) to stimulate both growth and oxidation rates of these organisms (see summary by Sharma and Ahlert, 1977) illustrates the importance of mixotrophic nutrition to these organisms. Specific aspects of medium formulation for the isolation of these organisms from the lacustrine environment will be discussed in Chapter 2.

The biochemistry and energetics are reviewed by Aleem (1977), Kelly (1978), Suzuki (1974) and Zumft and Cardenas (1969). Ammonia is initially oxidised to hydroxylamine, a reaction which involves molecular oxygen and the production of a proton (Bhandari and Nicholas, 1979). Under standard conditions this reaction is endergonic having a standard free energy change ( $\Delta G^{\circ}$ ) of approximately  $15 \text{ kJ. mole}^{-1}$  (Aleem, 1977). The oxidation of hydroxylamine to nitrite, via an unknown intermediate in which nitrogen has an oxidation state of +1, is mediated by electron transport systems and results in A.T.P. production. The P:O ratio for this oxidation is unknown. The standard electrode potential of the hydroxylamine/nitrite couple is +66 mV which suggests that electrons could couple at the flavoprotein or cytochrome b level in electron transport systems. This should result in a P:O ratio of 2, however, Aleem (1977) considers that the majority of electrons couple at the level of cytochrome c. Reports on hydroxylamine oxidoreductase have been given by Hooper et al (1978), Hooper (1978) and Hooper and Terry (1979).

Oxidation of nitrite to nitrate is direct and involves the oxygen atom of a water molecule. A reduced co-factor appears to couple to electron transport systems at the level of cytochrome c resulting in a P:O ratio of 1, (Aleem, 1977). Recent reports on the cytochrome composition of N. agilis are given by Cobley (1976), Yamanaka et al. (1979) and Chaudry et al (1980a, 1980b).

Various aspects of nitrification have been reviewed in Schlessinger, (1978), and Belser (1979) has reviewed the population ecology of

nitrifying bacteria with particular emphasis on soil populations.

The effects of environmental conditions on the activity of these organisms have been extrapolated from results obtained in culture studies, and frequently the activity of organisms in situ is discussed in relation to these values (Christofi, 1978). However, it is not clear to what extent results obtained with cultures would be applicable to natural situations. The effect of the activity of these organisms on the environment is, on the other hand, well established. The removal of ammonia is important because free ammonia (the concentration of which is dependent upon total ammonia concentration and pH) is toxic to fish (Alabaster and Lloyd, 1980) and can reduce the efficiency of chlorination of abstracted water supplies due to chloramine production. The occurrence of high nitrate concentrations, caused directly or indirectly by nitrification, in such supplies, can also cause public health problems (Nicholson, 1979). A further consequence of nitrate production from ammonia by nitrification is the increased oxygen demand within the ecosystem. Garland (1977) reported that nitrification accounted for up to 75% of the total oxygen demand in British rivers. If nitrification occurs to the extent that oxygen depletion becomes apparent the hydrogen ions which are produced during the oxidation of ammonia to nitrite, may affect the pH of the ecosystem. This will obviously depend upon the buffering capacity of the chemical environment but nitrification has been associated with pH decreases in the marine environment (Gunderson and Mountain (1973). Another product of phase I oxidation, nitrous oxide, has been implicated as a causative agent of adverse effects of the stratospheric ozone layer as discussed by Bremner and Blackmer (1978). Although it is generally a minor product, nitrous oxide has been observed in nitrifying marine systems (Cohen and Gorden, 1979), soil systems (Matthias et al, 1980) and in culture (Ritchie and Nicholas, 1972).

Recent reviews on this topic have been provided by Roswall, (1980), Bremner and Blackmer (1980) and Hahn (1979).

#### Heterotrophic nitrification

This phenomenon has been recognised for a number of years and a recent review by Verstraete (1975) discusses the organisms, substrates and products and the biochemical pathways which are involved. There are also more recent reports of this activity in soils and culture (Ishaque and Cornfield, 1976; Tate, 1977, 1980; Gowda et al, 1977; Castellui and O'Shanahan, 1977; Johnsrud, 1978, and Braunova and Bernatt, 1978). I am unaware of any reports on further work on the biochemistry of the various oxidations or the contribution, if any, of these oxidative reactions to the energy budget of heterotrophic nitrifying organisms. The significance of the process in natural environments is, as yet, unquantified and many arguments used to support the view that the process does occur in situ are not conclusive (see Verstraete, 1975). Heterotrophic nitrification is not readily demonstrated in natural samples and the reasons why such organisms should nitrify are purely speculative. Many of the end products are toxic, mutagenic or, at the other extreme, can act as growth factors (Verstraete, 1975). It is possible therefore that the production of these compounds enable the organisms to establish some competitive advantage within an ecosystem. Direct evidence for this is however, completely lacking.

The methylotrophic bacteria (methane oxidising organisms) have been shown to oxidise ammonia to nitrite in culture (O'Neill and Wilkinson, 1977; Dalton, 1977; Romanovskaya et al, 1976; Whittenbury et al, 1975) and further similarities between these and the phase I nitrifying organisms have been discussed (Hubley et al, 1975; Whittenbury and Kelly, 1978). By definition the energy producing metabolism of the methane oxidisers must be regarded as chemoorganotrophic and, as reduced carbon is required for growth (see Brock, 1977) they must also be regarded as

heterotrophs. However, due to similarities between carbon assimilation in these and other autotrophic micro-organisms Whittenbury and Kelly (1978) proposed that the definition of an autotroph be broadened to include the methylotrophs and other organisms assimilating single carbon compounds.

The methylotrophs, in common with many but not all heterotrophic nitrifiers, only oxidise reduced nitrogen to the oxidation state of nitrite. As most nitrifying systems produce nitrate as the dominant end product the occurrence of heterotrophic nitrification must be in association with a nitrite oxidising organisms. Recently Castignetti and Gunner (1980) have demonstrated such an interaction, in culture, between a nitrifying species of Alcaligenes and Nitrobacter agilis.

This brief discussion indicates that microbial ecologists, involved in nitrification research, are dealing with not only a specific process but also with different organism activities. The demonstration that certain bacterial species, isolated from the environment, are capable of performing reactions in culture does not mean that those reactions will occur in situ. In the following chapter I will survey the methods available for investigating the nitrification process and evaluate these for determining specific microbial involvements.

## CHAPTER 2

## A Review of the Methodology for Nitrification Research

## 2.1 Introduction

After his first isolation, in pure culture, of the lithotrophic nitrifiers in the early 1890's (Winogradsky 1890) it is reputed that Winogradsky returned to similar work forty years later and found that little progress had been made. Schmidt (1978) commented that now, another forty years on, many scientific advances had been made and there was widespread recognition of the importance of the nitrification process, however, there were many aspects of nitrification research that would still leave Winogradsky unimpressed. That was not to say that little effort had been made but that the process was, and still is, inherently difficult to study. This is due not only to the central rôle of ammonia and nitrate in a dynamic process (the nitrogen cycle), but also to the biochemical diversity of the organisms implicated in the process (see Chapter 1). Therefore many of the problems encountered in nitrification research are methodological and there is still a requirement for the classical nitrifiers of Winogradsky to be defined and quantified in relation to nitrogen oxidation. It is also necessary to define the ecological rôle, if any, of the heterotrophic nitrifiers, and methane oxidisers, in various ecosystems but particularly the freshwater environment. This is an attempt to review and discuss the methods currently available for the study of classical nitrifiers and to comment on the use of these methods as tools to investigate the possibility of other microorganism involvement.

Brezonik (1968) and Christofi (1978) broadly defined two categories of methods 1. Non-isotopic approaches . 2. Isotopic approaches.

## 2.2 Non-isotopic approaches

These methods do not involve the use of isotopic tracers and can be further sub-divided into (a) Direct microscopic observation (b) Culture techniques and (c) Activity estimates.

### 2.2.1 Direct microscopic observation

These methods are based on the identification or counting of stained or unstained bacterial cells directly in sample material. As mentioned above, the range of organisms implicated in oxidative nitrification reactions indicates that direct observation of samples, without some kind of pre-treatment, would be of little use in nitrifier identification. However, Johnson and Sieburth (1976) made use of the morphology of the intracellular cytoplasmic membranes of the classical nitrifiers (now used as a diagnostic criterion, Watson, 1974) to study nitrifier-like cells in actively nitrifying aquaculture systems. They listed the organisms known to have similar cytoplasmic membrane structures as the methylotrophic bacteria, the Cyanobacteria and the purple sulphur and non-sulphur bacteria. These organisms were eliminated on the basis of their non-cyst like colonies, thick cell walls and distinctive cell morphology plus the requirement for light respectively.

Studies of this type are always open to the criticism that they can only be used satisfactorily on pure cultures, or as Johnson and Sieburth (1976) point out, in environments where the organisms of interest are the dominant group present.

A direct observation method which involves pre-treatment of the sample is the Fluorescent antibody technique. This has been extensively employed, over the last 5 years, as a tool in the study of nitrifier ecology, particularly for investigating the nitrite oxidising bacteria (Fliermans et al, 1974; Fliermans and Schmidt, 1975; Rennie and Schmidt,

1977a, 1977b; Rennie, 1977, 1978; Belser and Schmidt, 1978a, b; Belser, 1979, Josserand and Cleyet-Marel, 1979; Kaltoff et al. 1979). The principle of the method is initially to isolate the organism of interest in pure culture and to prepare antibodies towards it, usually by inoculation into Rabbit. A fluorochrome is then attached to the antibody after its purification from the blood serum, and the subsequent fluorescent antibody (F.A) is used to investigate experimental samples. After exposure to the antibody the sample is viewed under epi-fluorescent microscopy and the cells which fluoresce are counted. FA's have been prepared against a number of ammonia oxidising (Belser and Schmidt, 1978a, b; Ward and Perry, 1980) and nitrite oxidising (Rennie and Schmidt, 1977a; Josserand and Cleyet-Marel, 1979) isolates. The success of the method depends on a) preparation of antibodies which will label all the organisms of interest in the sample and b) being able to distinguish bacterial cells from non-specifically labelled detritus present in the sample.

Fulfilment of the first objective depends on being able to isolate the ecologically dominant species from the habitat being studied, or preparation of antisera which will bind to all the species present. Obtaining pure culture isolates of lithotrophic nitrifiers is recognised as a difficult, and often time consuming, process; there is also evidence that the culture media presently in use may favour the isolation of only certain genera (Belser and Schmidt, (1978a)). It is therefore difficult, when embarking on an investigation, to predict the likely success of this approach. However, the specificity of several antisera thus far prepared has been tested (Belser and Schmidt, 1978b; Rennie and Schmidt, 1977a; Josserand and Cleyet-Marel, 1979). Of the phase I populations Belser and Schmidt (1978b) prepared antisera against seven strains of Nitrosomonas, five strains of Nitrosospira, three strains of Nitrosolobus



and one strain of Nitrosovibrio from various sources. Using these they found that cross reactions between genera were limited, and even within a genus the amount of cross reaction varied. This indicates that the use of F.A. to characterise the total population would be quite laborious as no single totally strain-specific F.A. can be produced. There was also an indication of between site differences within the same genus but exceptions to this have been observed (Belser 1979). Clearly if cross reaction can be demonstrated from serotypes isolated from different regions the application of this method would become more acceptable.

Until recently the problems associated with identifying the nitrite oxidising bacteria using F.A., in terrestrial ecosystems at least, were not thought to be as complex as for the ammonia oxidisers. Nitrobacter was the only genus which had been isolated and the two type species N. agilis and N. winogradskyi, were shown to be immunologically distinct (Rennie 1977) and not grouped as N. winogradskyi as in the most recent classification (Watson, 1974). Using F.A. for these species Rennie and Schmidt (1977) obtained evidence that the Most Probable Number counting technique grossly underestimated the phase II populations in soil, particularly at acidic pH. However, Kaltoff et al (1979) indicated that the nutritional basis of the culture used, for antiserum preparation, could affect the cross reactions obtained depending on whether the cells in the natural environment were growing autotrophically, mixotrophically or heterotrophically. In a natural situation mixotrophic nutrition would probably be the norm and unfortunately the reaction of antiserum from autotrophically grown cells was not tested on this type. These workers also showed strong cross reaction between autotrophically prepared antisera and autotrophically grown cells of the two species, a feature not apparent in the work of Rennie and Schmidt, (1977). This could highlight the nutritional basis of cells growing in situ. Furthermore Josserand and Cleyet-Marel (1979), using antisera prepared against A.T.C.C. cultures

of N. agilis and N. winogradskyi, found no cross reaction with two out of four nitrite oxidising isolates from French soils; nutritional differences were not responsible for the failure to react as all cells were grown autotrophically. Watson and Waterbury (1971) isolated and described two other genera of nitrite oxidisers from marine samples and as Nitrobacter appears to be fairly ubiquitous, the aquatic environment may contain more genera than was originally thought. Stanley and Schmidt (cited in Belser, 1979) have characterised at least seven more immunologically distinct strains of Nitrobacter.

The problem of distinguishing non-specifically labelled detritus, present in the sample, from bacterial cells, was discussed by Rennie and Schmidt (1977b) who outlined a protocol for F.A. methodology on soil samples which included the dispersal of cells from soil particles and subsequent separation of these two components. This system could easily be adapted for investigating sediment samples and possibly checked for accuracy by use of internal standards. Webb and Wiebe (1975) allowed microscope slides to be colonised in situ for four weeks prior to F.A. counts being made on these. Bohool and Schmidt (1973) followed the growth of Nitrobacter species in liquid culture by F.A. counts on membrane filters. Stanley et al (1979) used a similar system in a study on Nitrobacter in Lake Itasca. However, the reason for these authors using 0.45  $\mu\text{m}$  pore size filters is not clear as some cells would become embedded in the membranes and might therefore not be seen. Fliermans and Schmidt (1975) commented that moribund cells will be stained by F.A. and therefore would contribute to the count but not to in situ activity. In soils these dead cells would be rapidly scavenged but whether this would happen in other systems is not clear. It is also well established that starved or senescent nitrifier cells can survive for considerable periods in adverse environments (for example anoxic sediments, Christofi,

1978; Niewolak, 1970) and these cells will be counted although they are not active in situ. Fliermans and Schmidt (1975) overcame this problem by combining the F.A. technique with autoradiography. The sample was exposed to C-14 labelled bicarbonate prior to being exposed to the F.A. preparation. The subsequent use of epi-fluorescence and transmitted dark field microscopy identified the viable nitrifier cells. Although this method yielded very useful data it is probably too elaborate to use on a routine basis. In addition, soil particles were difficult to distinguish from 'developed' silver grains, but this could be overcome by the use of phase contrast microscopy. Clearly from this discussion it appears that F.A. techniques can be a useful tool for studies on the ecology of the classical nitrifying bacteria, particularly autoecological investigations where the great precision of the counts obtained (Belser, 1979) allows small population changes to be detected. However, total population studies have, up to present, been confounded by the lack of information on the different serotypes present in the environment. Information on these is dependent upon the isolation techniques used which will be discussed later. Over and above the microbiological difficulties, use of the method requires specialised facilities which are not always available to research workers (Schmidt, 1973).

The application of F.A. techniques to the study of other nitrifying organisms is possible; Schmidt (1973) reviews the historical development of this field of autoecological research and cites a number of studies involving heterotrophic soil microorganisms. Much of this early work was directed towards the nitrogen fixing organisms due to their importance in the global nitrogen cycle but could easily be applied to the Arthrobacter isolates found to be implicated in heterotrophic nitrification (Witzel and Overbeck (1979); Laurent (1971)).

## 2.2.2 Culture techniques

These methods rely on the fact that any viable nitrifying cells in the sample are capable of growth, or activity, under the cultural conditions provided. Growth, or activity, is recognised as a decrease in substrate concentration, increase in product concentration or production of visible growth. These techniques can be broadly divided into a) potential techniques and b) counting techniques.

### 2.2.2.a Potential techniques

As the title suggests these methods involve the measurement of the potential of the sample to nitrify, and not the actual activity occurring in the sample. The basis of the method is to incubate the sample and to measure, after timed intervals, the concentrations of either ammonia, nitrite, nitrate or possibly heterotrophic nitrification products. The nitrification potential is then expressed as the time to remove, or accumulate, a pre-determined amount of substrate or product respectively. The longer this time the lower the nitrification potential of the sample. Recent reports on the use of this method on sediments are found in Chen et al (1972a), Cavari (1977) and Christofi (1978) and on water in Kholdebarin (1977a, b); Cavari (1977) Christofi (1978), Swerinski (1977), Seppanen and Wunderluch (1970), Rho and Gunner (1978); Landner and Larsson (1973). Sediments are usually studied by obtaining a sediment and diluent slurry. Diluents which have been used include i) deionized water (Chen et al 1972a) ii) filter sterilized lake water (Cavari 1977) iii) aged sea water (Swerinski 1977 and iv) defined inorganic media (Seppanen and Wunderluch 1970). It is apparent that these additions remove the sample from in situ conditions, indeed in many cases nitrate would only accumulate if the sediment slurries were actively aerated (Chen et al 1972a; Christofi 1978) which could indicate that diffusion of substrates into particles was limiting activity (see Shieh and LaMotta,

1979). Cavari (1977) found that it was the lag period that increased and not the rate of oxidation, if the nitrification potential was low.

Because of the dynamic nature of the nitrogen cycle in such systems (Brezonik 1968) and the length of incubation used (14 - 40 days) it is likely that measurements of this type give little quantitative information on in situ rates of activity or nitrogen chemistry. This problem is further compounded by the incubation temperatures used; for example, Cavari (1977) sampled different depths of Lake Kinneret both during the stratified and isothermal periods and used an incubation temperature of 30°C throughout (Lake Kinneret however would have high in situ temperatures) and Swerinski (1977) used a temperature of 20°C whilst sampling the Kiel Bight between October and February. Christofi (1978) estimated his potential activities after incubation at in situ temperatures and found reasonable agreement between these and independently measured rates. However he did concede that the potential activity would be underestimated due to product utilization during incubation. If the products of nitrification by lithotrophs and heterotrophs are the same, and this can be the case (Verstraete, 1975), the potential method will not distinguish the processes. Chen et al (1972a), Tiedemann (1977) and Christofi (1978) overcame this problem by the addition of specific inhibitors for the lithotrophic nitrifiers (these are discussed in section 2.3.2.) and all found that nitrate accumulation was stopped indicating the involvement of a lithotropic<sup>h</sup> process. Cavari (1977) also stated that heterotrophic nitrifiers were not important in aquatic systems but forwarded no reasons, whilst Swerinski (1977) stated that there was not enough organic carbon in his samples to sustain heterotrophic activity (see Verstraete 1975). On this latter point Gode and Overbeck (1972), commenting on heterotrophic nitrification in culture, observed that nitrification was most pronounced in the stationary, or decline, phase of growth, when organic substrate is likely to be very low in concentration.

As mentioned above, sediment samples have to be aerated before nitrate will accumulate, indicating an oxygen limitation. Oxygen concentrations, sufficient to support aerobic activity, will only be present in the sediment surface layers, the actual depth of penetration being largely dependent upon the overlying oxygen concentration, the oxygen demand of the sediments and the degree of turbulence in the water column. This rarely extends deep into the sediment and therefore Christofi (1978) who sampled the top 5 cms of sediment and Chen et al (1972a) who did not define the depth of sediment sampled by his Eckman grab, may have organisms active under the experimental regime which could not be active in the natural environment. Undiluted water samples have also been examined using the potential technique. However, the substrate concentration has often to be increased (Cavari 1977) which again removes the sample from in situ conditions.

From the above discussion it becomes apparent that although this method is simple to perform and requires little in the way of specialized equipment, its use for quantitative studies is limited and is probably best employed in the comparison of samples or to measure the effects of different environmental variables on the nitrification process, as used by Rho and Gunner (1978) and Kholdebarin (1977a, b). However, it should be noted that Cavari (1977) found a good correlation between the lag period, before nitrification commenced, and the numbers of nitrifiers present in the sample (dilution of a pure culture). Srinath et al (1976) and Hall and Murphy (1980) also attempted to estimate nitrifier numbers, or biomass, in natural situations, by using parameters estimated from pure cultures. It is not clear, as yet, how activities in pure culture can be related to activities in situ and therefore these results should be interpreted with caution, as pointed out by the respective authors. Belser (1979) has discussed the disparity between pure culture and in situ yield estimations for the lithotrophic nitrifiers in the soil ecosystem.

### 2.2.2.b Counting techniques

These techniques have predominated in microbial ecological studies ever since such studies began, in spite of the known limitations of the methods (Schmidt 1973; Buck, 1979). The methods involve the plate count and Most Probable Number (MPN) techniques.

#### i) Plate counts

The solidifying agents for plate counts are usually inorganic gels e.g. silica gel (see for example Collins, (1969); Tu, (1973)) or purified agars e.g. Difco Noble agar or purified Merck agar (Soriano and Walker, 1968). These have low concentrations of organic impurities and therefore suppress the growth of heterotrophic contaminants. Gould and Lees (1960) also added antibiotics as a further measure. Winogradsky (1890) used silica gel, but this was due to the widely held belief that organic material was inhibitory to the lithotrophic nitrifiers. It is now more generally accepted that oxygen limitation due to rapid heterotrophic activity causes the inhibition, although some organic compounds are known to have a direct inhibitory action (Matin 1978). Even in the presence of inert solidifying agents Schmidt (1978) states that plating techniques are virtually useless due to the introduction of organic materials with the inoculum. These permit the more rapid growth of non-nitrifying heterotrophs and consequently make counting difficult. Despite the objections the methods which have reportedly been successful are reviewed.

Tu (1973) compared silica gel plate counts with MPN counts and concluded there was no significant difference between the results of the two methods. The colonies were checked for nitrifying ability, by inoculation into a liquid medium, and it was found that 98 and 96% respectively, of the presumptive phase I and II colonies, nitrified. Ashworth (1973) also used silica gel but used the pour plate procedure after mixing calcium carbonate with the gel prior to solidification.

This carbonate was dissolved by the acid produced by nitrifier colonies and showed as clear areas in the plate. Similar methods involve spreading a layer of  $\text{CaCO}_3$  over the surface of the plate (Walker 1975) or the addition of phenol red to the medium as a pH indicator (Soriono and Walker 1968). These latter authors reported that the nitrifier colonies were small (< 0.2 mm diameter) and had to be removed with a micro manipulator; this would make routine counting difficult. Finstein and Bitzky (1972) filtered water samples through 0.45  $\mu\text{m}$  membranes and placed these on glass wool pads soaked in ammonia containing medium. After incubation the filters were thoroughly rinsed and exposed to fresh medium for 15 minutes. A nitrite colourimetric spot test was used to demonstrate the colonies which, because of the lack of diffusion, appeared as discrete spots. Kuniki-Goldfinger (1967) (cited in Kuniki-Goldfinger 1974) described a semi-continuous culture apparatus developed for measuring those nutrients which were assimilated from water samples. This involved the filtering of a sample through a membrane filter which was then incubated using the filter sterilized sample as medium (Kuniki-Goldfinger, 1974). Lukaszewicz (1968) (in Kuniki-Goldfinger, 1974) reported that this technique proved useful for the isolation and enumeration of nitrifying organisms.

The only reported plate counts of non-specific heterotrophic nitrifiers was by Christofi (1978) but this method was abandoned in favour of an MPN procedure.

#### ii) Most Probable Number counts

(a) General. This is the most usual counting method used in the study of the microbiology of nitrification and is an indirect statistical count the theory of which is described by Cochran (1950).

The use of test tubes, for MPN counts is often time consuming and, depending on the experimental design, can take up considerable space. Curtiss et al (1975) reduced this space by using Repli dishes (Sterilin),



with 5 replicates per dilution, and Rowe et al (1977) reduced this even further by using micro-titer plates (Sterilin) which gave 8 replicates per dilution; the dilution interval, in this latter system, can be less than ten-fold e.g. 1:4 or 1:2, being easy to perform using the 8 channel piston pipettes now available. (See Jones (1979) for sources of relevant MPN tables for 1:10, 1:4 and 1:2 dilution intervals).

The scoring of positive tubes in MPN counts is usually done by the appearance or disappearance of nitrite for phase I and II organisms respectively. Nitrite is the chosen test substrate because it can be detected with speed and sensitivity. Care must be taken in the interpretation of apparent negative results. The absence of nitrite does not imply a negative result for phase I organisms because the nitrite formed could have been further oxidized to nitrate by phase II organisms present in the initial inoculum, (these organisms are known to survive for long periods in the absence of their specific substrates). Therefore if nitrite is absent a nitrate spot test should be employed to avoid false negative results. An alternative approach was used by Curtiss et al (1975) who added fresh ammonium sulphate 24 hours before the spot tests were made to ensure nitrite would be present in the positive tubes. For phase II organisms absence of nitrite is not always a good indication of positive tubes as inorganic nitrogen, particularly at the low concentrations normally used for nitrite, is readily available to heterotrophic prokaryotes also growing in the medium. Therefore a spot test for nitrate should also be made.

It is generally accepted that any MPN technique is based on two assumptions:- a) that one cell (the theoretical minimum) will initiate growth in the medium and b) the medium will support the growth of all the organisms of interest present in the inoculum. With regards to the first point there is some evidence that nitrifiers, in the natural environment, are associated with particulate material (Curtiss et al, 1975;

Paerl et al 1975) although they have been successfully cultured in particle free media (Engel and Alexander, 1958; Goldberg and Gainey 1955). Therefore to obtain maximal counts the particle-bacterial association has to be disrupted. Christofi (1978) treated sediment samples with sodium tripolyphosphate to separate the bacteria from particles prior to MPN counts, whilst Vanderborght and Billen (1975) used homogenization, although they did not subsequently use counts.

(b) Media. There are numerous media formulations in the literature and an attempt is made here to establish the relevant points about medium formulation and the environmental conditions of incubation which could directly affect the MPN methodology and results.

The nutritional requirements of the lithotrophic nitrifiers are fairly well established (Painter 1970, 1977) and most media will supply the essential nutrients either as direct additions, or in sufficient quantities as contaminants. However in the following pages I draw attention to certain gross features of each medium.

### 1. Salinity

It seems unusual to discuss salinity in a thesis dealing with freshwater microorganisms. However sodium chloride is a common constituent of media used for freshwater studies (for example Curtiss et al 1975) and therefore a discussion on the tolerance of these bacteria to different saline concentrations is relevant.

There have been numerous studies which have investigated the differing effects of salinity on marine and terrestrial isolates of nitrifying bacteria, (Thomsen (1910); Yankovitch and Yankovitch (1973); Vargues and Brisou (1963), Watson (1963)). Without exception these workers observed that the marine isolates, although showing distinct morphological similarities to terrestrial forms, were more tolerant of high sodium chloride concentrations than the latter organisms. This was either displayed as terrestrial isolates having longer lag periods, indicating

a certain amount of adaptation, or a complete failure to grow. Yoshida et al (1967) studied the distribution of nitrifying bacteria in river and coastal sediments using two media, one for marine samples with 100% sea water, and the freshwater medium containing only 0.005% sea water.

Finstein and Bitzky (1972) investigated the relationship of phase I organisms to marine salts using a seawater and freshwater media. Generally marine isolates grew in both media but, with one exception, freshwater nitrifier enrichments could only be performed with the freshwater medium. The authors concluded that the organisms' requirements reflected their environment although slow adaptation to saline conditions can occur.

Koops et al (1976) isolated Nitrosococcus mobilis from slightly brackish water. The isolated bacteria would only tolerate 1.1% sea water and the sodium, in the medium, could not be replaced by K, Mg or Ca.

From this brief review it becomes apparent that the choice of isolation medium is dependent upon the type of samples being investigated and salinity has been shown to be one of the more important variables affecting nitrifier distribution in estuaries (Refendran and Venugopalan 1977).

#### Temperature of Incubation

From the literature it is observed that most MPN determinations are carried out at temperatures between 20°C and 30°C which are often considerably above the normal temperature of the sample. Anderson et al (1971) using soil samples from different prevailing climatic temperatures found high temperature adapted isolates were not active at low temperatures, whilst low temperature isolates could be more active at higher temperatures (see also Monib et al, 1979). Chen et al (1972a) using a 'potential technique' found greater activity at 25°C than at 10°C and this was confirmed by Christofi (1978). Niewolak et al (1978) reached the same general conclusion but also found some samples which were more active at the lower in situ temperatures. Therefore the temperature of incubation should create no problems unless it is lower than the sample temperature.

Increases in temperature decrease the solubility of gases and as oxidation of 1 mg  $\text{NH}_4^+$ -N to  $\text{NO}_3^-$ -N requires 4.57 mg oxygen adequate aeration must be ensured.

#### Medium pH.

It is generally recognised that the lithotrophic nitrifiers are most active at pH values on the alkaline side of neutrality and that optimal activity is not always associated with optimal growth (Painter 1970). However, many of these studies were carried out on pre-cultured cells and it is possible that these had become adapted to the pH of the isolation medium. Table 2.1 lists the optimal pH range found for a number of pure cultures and the pH of the initial isolation medium.

Much of the work on the effect of medium pH on the isolation of nitrifiers has been done on soil (Ul'yanova (1961) Belser and Schmidt (1978), Morrill and Dawson (1967)) and it is not clear if these results can be extrapolated to the aquatic environment. Generally the optimal pH for isolation reflected the pH of the environment. Rennie and Schmidt (1977), using F.A. techniques, found the counting efficiency, of one medium for *Nitrobacter* enumeration, decreased as the soil pH decreased. Walker and Wickramasinghe (1979) could only isolate Nitrosospira, Nitrosolobus and one species of Nitrosovibrio from acid tea soils (pH 4.0 - 4.5) of Sri Lanka and Bangladesh, which may indicate a different species dominance in these soils, the pH of the isolation medium was not reported.

The effect of pH on the response of the nitrifiers is closely related to the substrate concentration (see below) however more work is required on the effects of medium pH on the primary isolation of these organisms.

#### Substrate concentration

Both phase I and II organisms are inhibited by high concentrations

Table 2.1 The relationship between the pH of the initial isolation medium and the experimentally determined optimum pH for activity of the Nitrobacteriaceae.

Organism	Isolation pH	Optimum pH	Reference
Nitrosomonas	8.5	8 to 9	Hofman and Lees (1953)
Nitrosomonas	7.9	7.5 to 8.0	Loveless <u>et al</u> (1968)
Nitrosomonas	8.0 to 8.5	8.0 to 8.5	Buswell <u>et al</u> (1954)
Nitrosomonas	8.5	8.3 to 9.3	Meyeroff (1917) (in Painter 1970)
Nitrosomonas	8.3 to 8.6	8.5	Lees (1959)
Nitrosomonas	7.2 to 9.0	8.0	Engel <u>et al</u> (1958)
Nitrobacter	7.2 to 8.2	7.8	Lees <u>et al</u> (1957)
Nitrobacter	7.3 to 8.4	7.6	Boon <u>et al</u> (1962)

of their own substrate as well as that of the other organism (Painter 1977). The inhibitory action seems to be caused by free ammonia and nitrous acid (Wong-Chong and Loehr (1978). The actual concentration of these species is dependent upon pH and the concentrations of ammonia and nitrite. Anthonisen et al (1976) produced a diagram of concentration and pH for both active species and the patterns of inhibition which will be obtained. From the general range of media pH used for isolation it is most likely that free ammonia is the toxic agent: if nitrite is added at levels to give inhibitory concentrations of free nitrous acid it is likely that the nitrite alone would itself be toxic. Interestingly Suzuki et al (1974) found evidence which suggested that free ammonia was the actual substrate for phase I oxidation. Little work is available on the effect of substrate concentrations on the isolation of lithotrophic nitrifiers although Lozinov and Ermachenko (1959) found maximal nitrite production, by a pre-grown phase I isolate, with  $140 \text{ mg N l}^{-1}$  (pH 7.7). The range of substrate concentrations found in the literature is large and some are given in Table 2.2.

#### Length of incubation

Matulewich et al (1975) quote incubation time used for MPN counts of both phase I and II organisms from 21 to 90 days. Ideally the incubation time should be of a duration which allows the growth of all viable cells. Using two defined media, and samples from different environments, they reported phase I counts to increase up to 55 days (range 20 - 55) whilst maximal phase II counts were not obtained after 113 days incubation. Clearly it is not practical to use such long incubation times on a routine basis. It is possible however that the range of incubation times could be reduced if the medium was formulated to resemble, more closely, the conditions found in the sample since the above discussion indicates that there is considerable adaptability within the Nitrobacteriaceae.

Table 2.2 The range of substrate concentrations used, in media for the isolation of ammonia oxidising chemolithotrophic bacteria.

Reference	Ammonium salt	[N] mg n l <sup>-1</sup>	Environment
Tu (1973)	Sulphate	106	Soil
Skerman (1967)	"	140	
Sinha (1968)	"	530	Soil
Alexander & Clark (1965)	"	1 and 530	"
Soriano & Walker (1968)	"	106	"
Finstein & Bitzsky (1972)	"	318	Various
Goldberg & Gainey (1955)	"	212	Soil
Wezernak & Gannon (1967)	"	1	Sewage
Ul'Yanova (1959)	Various	11,200 to 280	Soil
Vargues & Brisou (1963)	Sulphate	2758	Marine
Carlucci & Pramer (1968)	Various	212	
Gode & Overbeck (1972)	Sulphate	55	Freshwater

iii) General observations on the culture of lithotrophic nitrifiers

Schmidt (1978) states that it is difficult to assess the accuracy, and sensitivity, of MPN methodology for nitrifiers because no standard exists. Whilst this is broadly true (no MPN method has a real standard) work with F.A. has given certain indications as to its limitations.

Rennie and Schmidt (1977) using pure cultures of N. winogradskyi and N. agilis found F.A. counts to closely follow Petroff-Hauser counts whilst MPN counts, except for one instance, were a factor of  $10^2$  lower. These results were obtained from pre-cultured cells and one would expect counts directly from the environment to be even less efficient. Belser and Schmidt (1978) compared MPN with F.A. counts (for Nitrobacter) from soils of varying pH. The MPN counts were always lower and the difference was pH dependent, being  $10^2$  at pH 7.2 and  $10^4$  at pH 4.7. It is worth noting that only N. agilis and N. winogradskyi F.A.'s were used and if the findings of Kaltoff et al (1979) and Josserand and Cleyet Marel (1979) (see pages 18,19) are generally applicable the MPN counting efficiencies could be even lower than expected. Webb and Wiebe (1975) could not enrich phase II organisms in samples from a coral reef but F.A. counts of Nitrobacter revealed  $10^6$  bacteria sp.  $\text{cm}^{-1}$  (the medium formulation was not given). Stanley et al (1979) obtained counts of Nitrobacter, in the water column of lake Itasca, to range between 100 and  $1000 \text{ ml}^{-1}$ , unfortunately MPN estimates were not attempted, and it is not possible to compare these results with those from other lakes.

The efficiencies of phase I counts are less clear, due to the difficulties with F.A. techniques described in section 2.2.1. Belser and Schmidt (1978a) compared four media, inoculated with exponentially growing cultures, and found high counting efficiencies, but the incubation times required to reach maximal counts varied greatly. Johnson and Silburth (1976) state that the usual cultural procedures could fail to detect the ecologically important nitrifiers which may not respond to,



or be able to compete in, the cultural conditions employed.

Clearly there is much to learn about the culture and counting of these ecologically important organisms and a major area for future research should be the isolation and characterization of the nitrifiers. Perhaps once this is achieved the application of F.A. techniques may be used to follow subtle population fluctuations and answer fundamental questions regarding their ecology.

#### iv) Culture of heterotrophic nitrifiers

One of the most frequently used arguments in favour of the occurrence of heterotrophic nitrification is that the numbers of lithotrophic nitrifiers detected in samples cannot account for observed increases in nitrate. This has been discussed in detail, for soils, by Belser (1979). From the above discussion of MPN methodology, where it appears that, phase II populations at least, are underestimated, this type of reasoning must be questioned. Arguments of this type can be found in Isirimah et al (1976), Gode and Overbeck (1972) Tate (1977) and Ishaque and Cornfield (1976).

There have been relatively few counts of heterotrophic nitrifiers in aquatic systems. Of the few which have been reported an MPN technique was used. These will be discussed further in the next Chapter.

### 2.3 Activity Estimates

The natural microbial environment is a dynamic system and, as the nitrogen cycle occurs intact in a variety of ecosystems it follows that inorganic nitrogen analysis on samples may not reveal any information on the rate of nitrification 'in situ'. Finstein et al (1977a) found no chemical evidence for nitrification during the flow of the Passaic River but microbiological investigations revealed extensive and active nitrifying populations. Despite this a number of reports are available in which nitrification is estimated by the change in nitrogen chemistry with downstream flow of river water. Ruane and Krenkel (1977) state that

this provides the best estimate of nitrification as the enclosure of samples, and the observed lag in the onset of nitrification, in sample bottles is not representative of the natural situation. These authors estimate the downstream travel of the body of water from average flow velocities. Curtiss et al (1975) used radioactive Bromine to label a specific body of water, however neither of these studies take account of diffuse non-point, or point, sources of nitrogen entering the river within the sampling reach. Remacle and Deleval (1978) point out that the river section must be free of such inflows in addition to the restriction of all boat traffic. These authors also empounded river water in plastic bags to follow changes in nitrogen chemistry, but as nitrification in rivers is probably primarily associated with the stream bed (Curtiss et al, 1975; Finstein et al, 1978; White et al, 1977; Gujer, 1978) this method may underestimate the effects on water chemistry. White et al (1977) used an equilibration chamber with water and sediments in contact, but the absence of flow within these chambers could interfere with various mass transport phenomena.

### 2.3.1 Nitrification inhibitors

Reliable estimates of in situ activity can only be obtained if:

a) the total flux of an inorganic nitrogen species is measured, or b) the microbial activities affecting the concentration of a nutrient are measured (for example see Brezonik 1968). More often a compromise is employed where chemical evidence is supplemented with specific microbiological data (Tiedemann 1977).

The first approach has been attempted in a number of nitrification studies, particularly in the terrestrial environment, and has been facilitated by the development of a number of compounds which specifically inhibit the lithotrophic nitrifiers. This development was accelerated by the increased pressure on agricultural practices in the early 1960's, and the awareness of the economic advantages of preventing the oxidation of cationic, and

readily absorbed, ammonia, to anionic, and easily leached, nitrate in the soil. It is also possible that these inhibitors reduce the nitrate loading in surface and subterranean water supplies, so reducing cultural and public health problems. Many inhibitors are now available and a full bibliography is given in the Commonwealth Bureau of Soils: Nitrification inhibitors 1969-1977. These compounds are particularly useful tools in nitrification research as the activity of lithotrophic nitrifiers can be estimated by comparison of various parameters in inhibited and uninhibited samples. Before discussing the parameters which can be used, the inhibitors which have been used in the nitrification literature will be reviewed briefly.

Two requirements for any inhibitor for research use are that: 1) it must prevent the growth and activity of the target organism(s) and ii) it must be specific to its target organism(s). Of the many inhibitors recognised only four have been utilized in ecological studies, N-Serve (Dow Chemical Co.), Allylthiourea, chlorate and ammonia.

#### 2.3.1(a) N-Serve

This inhibitor (2-chloro-6-trichloromethyl Pyridine) is also known under another trivial name, Nitrapyrin, and is the one most frequently used in ecological studies. The site of inhibition has been shown to be the initial oxidation of ammonia to hydroxylamine (Campbell and Aleem 1965). Inhibition by  $0.2 \mu\text{g ml}^{-1}$  was reversible by addition of  $6 \times 10^{-4}$  M cupric ions. This is surprising as Hooper and Terry (1973) grouped this compound with nitrification inhibitors which interact with enzymes or heme proteins, and not in the group of chelating agents. Total inhibition of nitrification is achieved by preventing the production of nitrite, the substrate for phase II organisms. Belser (1977) has identified nitrite production from nitrate but the ecological significance of this reaction in aquatic systems is unknown. The specificity of N-Serve has been well investigated and Table 2.3 shows the concentrations shown to be active

Table 2.3 Minimum concentrations of N-Serve reported to be inhibitory towards, or used to inhibit, various microorganisms.

Minimum inhibitory [N-Serve] mg.l <sup>-1</sup>	Test organisms	Environment	Reference
10.0	Phase I nitrifiers	Soil	Bundy & Bremner 1973, 1974
5.0	" "	Marine sediment	Billen, 1976 Vanderborcht & Billen 1975
0.01	" "	Brackish water and sediments	Cavari 1977
0.2 to 2.0	" "	Marine	Webb & Wiebe 1975
1.3 to 5.0	" "	Soil	Hickisch & Kopcanova 1977
1.0	" "	Freshwater sediments	Chen <u>et al</u> 1972a
5.0	" "	Freshwater	Somville 1978
	" "	Marine	Indrebø <u>et al</u> 1979
20.0	" "	Marine	Miyazaki <u>et al</u> 1975
1.0	Nitrosomonas europaea	Soil	Shattuck & Alexander 1963
> 20.0	Nitrobacter (agilis)	Culture	Campbell & Aleem 1965
> 20.0	Aquatic Hetero- trophic bacteria	Freshwater	Somville 1978
5.0	Sulphate reducing bacteria	Culture	"
> 10.0	Various Hetero- trophic microorganisms	Culture soil	Laskowski <u>et al</u> 1975
1,200.0	Soil microorganisms	Soil	Goring 1962
> 14.0	Denitrifying organisms	Soil	Mitsui <u>et al</u> 1964
> 10.0	Various micro- organisms	Culture	Shattuck & Alexander 1963
> 50.0	Denitrifying bacteria	Soil	Cribbs 1978

> 50.0	Denitrifying bacteria	Culture	Henninger & Bolag 1976
> 10.0	Heterotrophic bacteria	Culture	Billen 1976

Further references can be found in the text

against various test organisms, also included are the concentrations used in nitrification studies. Direct comparison of these data is not possible due to the different environmental conditions used but certain trends can be noted. Firstly, the concentrations found to inhibit the general bacterial flora are greater than those which inhibit the susceptible organisms (phase I nitrifiers, sulphate reducing bacteria and probably methane oxidising bacteria). However, both sets of figures cover wide ranges which could, in part, be due to different characteristics of the sample, or the incubation conditions used. For example Goring (1962); Hendrikson and Keeney (1979a) and Bremner et al (1978) found N-Serve to be absorbed by organic molecules and surfaces in soils, thus reducing the effective concentration, (there is no reason to assume that water samples would behave any differently). Hendrikson and Keeney (1979a) also noted that chemical hydrolysis of N-Serve, to chloropicolinic acid (which is inactive in controlling nitrification, Laskowski et al, 1975) is temperature dependent (see also Touchton et al 1979) but pH and sorption to organic material has no effect on this rate of hydrolysis. The observations of Hendrikson and Keeney (1979b), on the changing effectiveness of N-Serve in inhibition of nitrifiers at different pH levels is therefore a complex phenomenon. Bhuiya et al (1976) report that Nitrosolobus isolates are more susceptible than Nitrosomonas isolates, to N-Serve and so the predominance of Nitrosolobus species in acid soils (Walker and Wickramasinghe 1979) may, in part, explain this pH dependence.

N-Serve is virtually insoluble in water and therefore most workers prepare solutions using organic carrier solvents, although Bremner et al (1978) report preparation of an aqueous solution by shaking in a stoppered bottle for 24 hours. For example, Henniger and Bollag (1976) used ethanol, Laskowski (1975) used diesel oils, Belser and Mays (1980) used Dimethyl sulphoxide, others have used acetone. A full list of solubility properties is given by Goring (1962) but care must be taken in application as the

solvents used can also inhibit various nitrogen transformations by microorganisms (Bremner et al, 1978). Unfortunately this effect has not been investigated in any of the studies which use N-Serve, however appropriate controls are always performed (Billen 1976, Somville 1978).

The susceptibility of the methane oxidising bacteria, to N-Serve, is expected due to the similarities in morphology and biochemistry to the phase I nitrifiers (Whittenburg and Kelly, 1977; Suzuki et al, 1976). However, the sensitivity of the sulphate reducing bacteria is more puzzling as cytochrome b is the terminal electron acceptor, yet Campbell and Aleem (1965) found N-Serve to inhibit cytochrome oxidase of the Nitrosomonas respiratory chain.

N-Serve should provide a useful tool for elucidating lithotrophic or heterotrophic involvement in nitrification (Shattuck and Alexander, 1963) and has been used for this purpose in a number of studies (Chen et al, 1972a; Chistofi, 1978; Isirimah et al, 1976).

### 2.3.1(b) Allylthiourea (A.T.U.)

In reviewing the use of A.T.U. I will also refer to work using the closely related thiourea compounds. Campbell and Aleem (1965) found that complete inhibition of ammonia oxidation by Nitrosomonas, could be achieved by  $0.6 \mu\text{g ml}^{-1}$  A.T.U. and the site of inhibition was the initial oxidation of ammonia to hydroxylamine. This is the same site as N-Serve inhibition, but the authors concluded that the mechanisms were different as the action of A.T.U. could not be reversed by the addition of cupric ions. Mahli et al (1979) obtained similar results for the inhibition caused by  $0.5 \mu\text{g ml}^{-1}$  Thiourea. These data are again unusual, as Hooper and Terry (1973) attributed the ability of A.T.U. to inhibit nitrification to its metal binding properties. However, not all chelating agents inhibit nitrification (Sahrawat 1978). Hofmann and Lees (1953), Campbell and Aleem (1965) and Mahli et al (1979) reported that  $0.5$  to  $0.6 \mu\text{g ml}^{-1}$  Thiourea compounds were completely inhibitory to Nitrosomonas in culture;

this is 2.5 times greater than the required inhibitory concentration of N-Serve under the same conditions (Campbell and Aleem 1965), Hays and Forbes (1974) reported this difference to be an order of magnitude in soil perfusion solutions. Mahli et al (1979) reported that up to  $120 \mu\text{g ml}^{-1}$  Thiourea was required to completely inhibit soil nitrification. These are indications that A.T.U. may be less effective than N-Serve for inhibition of nitrification. The great advantage of A.T.U. is that it is soluble in water (saturated sol<sup>n</sup>. = 3% W/V or  $30 \text{ g.l}^{-1}$ ) and the possibility of side effects from organic solvent carriers, used with N-Serve, is thus eliminated. Table 2.4 lists the concentrations and use of A.T.U. in nitrification studies and it becomes apparent that there is little information on i) the effects of sample composition on its efficiency as an inhibitor ii) its effect on the general microbial flora and iii) its specificity. Lure'e and Odaryuk (1975) reported that  $0.5 \mu\text{g ml}^{-1}$  thiourea compounds did not affect the oxygen uptake of the saprophytic microflora and Hubly et al (1975) reported that  $1.0 \mu\text{g ml}^{-1}$  A.T.U. caused a 75% reduction in methane oxidation by Methylosinus trichosporium drawing attention to the similarity between this inhibition and that of phase I nitrifiers.

### 2.3.1(c) Chlorate

Painter (1977) reported that chlorate was inhibitory to Nitrobacter apparently after it first decomposed to chlorite. Belser and Mays (1980) reported that chlorate was specific for phase II organisms. When chlorate was added to a nitrifying sediment slurry, at 10 mM concentrations, nitrite accumulation continued at a constant rate whereas addition of  $20 \mu\text{g ml}^{-1}$  N-Serve caused nitrite accumulation to cease. They also found that chlorate decreased  $V_{\text{max}}$  and increased  $K_m$  (for nitrite) therefore the amount of inhibition obtained, with the same amount of chlorate, is dependent on the initial nitrite concentration of the sample. However, Schwert and White (1974) reported that 5 mM chlorate inhibited ammonia oxidation in an equilibration chamber containing water and sediments from a heated stream;



Table 2.4 Minimum concentrations of A.T.U. reported to be inhibitory to lithotrophic nitrifying organisms and one species of methane oxidising bacteria

Minimum inhibitory [A.T.U] mg l <sup>-1</sup>	Test organisms	Environment	Reference
0.5	Nitrosomonas	Culture	Malhi <u>et al</u> 1979
0.5	"	River water	Schornick & Ram 1978
0.5	"	Waste water	Lur'e & Odaryuk 1975
50.0	Phase I nitrifiers	Soil	Sahrawat 1978
0.2	" " "	River water	Tiedeman 1977
1.0	" " "	River sediments	Cirello 1975
1.16	Methylosinus trichosporium	Culture	Hubley <u>et al</u> 1975

Many other investigators have used A.T.U. but do not state concentration e.g. Garland 1976.

the reasons for this are unclear (see Belser and Mays 1980).

Cirello et al (1979) added  $10^{-5}$  molar chlorate and ammonia to river water; the inhibitor delayed the loss of nitrite from the water column, with time of travel downstream, for two days. The use of chlorate as an inhibitor has the disadvantage that it is also inhibitory to the enzyme nitrate reductase B, present in a large number of facultative anaerobic bacteria (Stouthamer 1976). Chlorate is reported to be only slightly inhibitory to phase I organisms but different genera may vary in their sensitivity.

#### 2.3.1(d) Ammonia and Urea

Roch and Kaffka (1972) used  $5.35 \text{ g NH}_4\text{Cl l}^{-1}$  ( $1.4 \text{ g N l}^{-1}$ ) to suppress nitrification in B.O.D. tests on the River Elbe, Germany. Ruane and Krenkel (1978) used urea for the same purpose but did not state the concentration. Free ammonia is known to be toxic to phase II nitrifiers (Anthonisen, 1976; Oertli, 1972). The levels of free ammonia will depend on ammonium ion concentration and pH of the samples. Langowska and Moskal (1974) exposed pure cultures of phase I (pH 7.7) and II (pH 8.0) organisms to various levels of ammonia and urea for 24 hours before testing activity. They found that  $0.5 \text{ g l}^{-1}$  ammonia-N completely inhibited both organisms whereas  $0.1 \text{ g ammonia-N l}^{-1}$  had no effect;  $10 \text{ g l}^{-1}$  and  $26 \text{ g l}^{-1}$  urea reduced the activity of phase I and II organisms respectively.

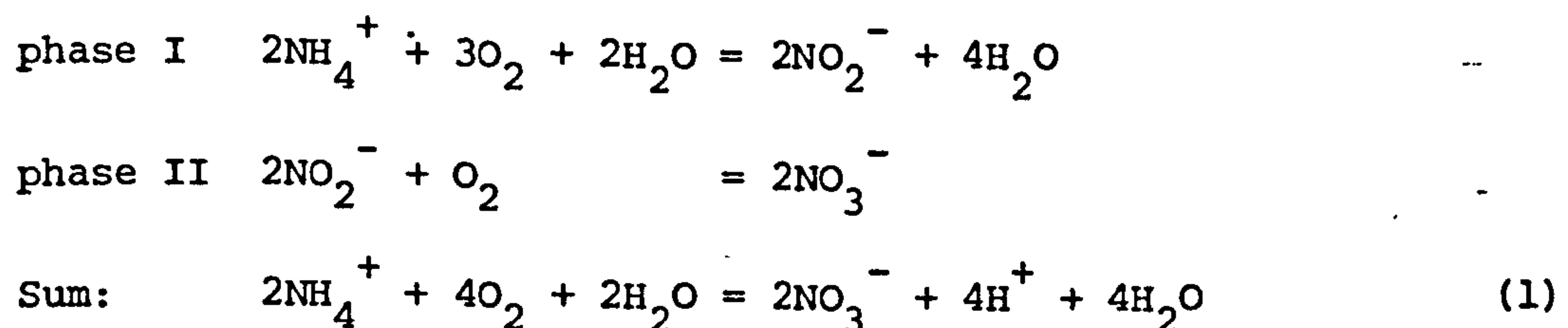
The addition of ammonia and urea to samples may stimulate the activity and growth of the heterotrophic microflora and therefore these inhibitors are probably of limited use in quantitative ecological studies.

#### 2.3.2 Use of inhibitors to estimate nitrification rates

In the following discussion on the use of inhibitors to estimate rates of nitrification it is assumed that the samples are enclosed in a container for a period of time sufficient to allow detectable changes to occur. During this incubation period the bacterial community may change and will therefore differ from the original population. This 'bottle

effect' has to be kept to a minimum (by control of time and temperature) if realistic in situ measurements are to be made. It should also be noted that both Brezonik (1968) and Christofi (1978) have obtained different estimates of nitrification rates by changing the volume of sample incubated. Generally planktonic populations are more susceptible than benthic populations to the bottle effect and broad outlines of the effects of time and temperature for planktonic populations are given by Jones (1977).

With the ability to almost selectively inhibit the lithotrophic nitrification process it is possible to estimate rates of change of the chemical species involved in the presence and absence of inhibitors. The equations for nitrification are represented by:-



Ammonia, nitrate and oxygen are suitable candidates for estimation.

### 2.3.2(a) Oxygen

This is used in the water supply industry to separate the carbonaceous and nitrogenous oxygen demand (B.O.D. and N.O.D. respectively) using the inhibitor A.T.U.

For ecological studies it is advantageous to convert the rate of oxygen uptake to that of nitrogen oxidised. From the stoichiometry of equation 1 oxidation of 1 g ammonium to nitrate (both as nitrogen) requires 4.57 g of oxygen. Wezernak and Gannon (1962) found the measured values to be 4.33 g oxygen due to some oxygen production in carbon dioxide fixation, although this has been questioned by Poduska (1973). The error in using either value is less than 10% which is sufficient accuracy for

most ecological measurements. Ruane and Krenkel (1978) criticised the use of this technique because nitrification often showed a lag in bottle samples filled with river water (but the benthos was the primary site of nitrification in this study). Further examples of this methodology are given in Roch and Kaffka (1972); Schornik and Ram (1978); Garland, 1976 and Cirello et al 1979.

#### 2.3.2(b) Ammonia and Nitrate

Of the methods available the estimation of these chemical species are probably the easiest to perform because of the simplicity of the chemical determinations and the absence of any need to exclude oxygen. Even if processes which utilize nitrate are dominant, rates of nitrate production can still be detected (Webb and Wiebe, 1975; Schwert and White 1974).

The usefulness of any of these chemical techniques is determined by the precision of the particular determination, the activity of the sample and the length of incubation which can be tolerated. This is discussed in relation to the present work on page 127-128.

#### 2.4 Other methods

Recently Sharma and Ahlert (1977) advocated that the potential of biochemical assays (identifying specific enzymes) should be investigated to enumerate, or estimate the activity of the nitrifying bacteria. No enzymes were specified but due to the ubiquity of the main metabolic pathways in the prokaryotes, presumably the specific energy producing enzymes of these organisms were in mind. Cell free ammonia oxidation has been reported (Watson et al, 1970; Suzuki and Kwok, 1970, both cited in Suzuki, 1974) after preparation of cell free extracts using a French pressure cell. Hydroxylamine oxido-reductase (E.C. 1.7.3.4) (Hooper and Terry 1979) is also becoming well characterized and does not appear to

require a particular membrane configuration for activity (Suzuki 1974) although ammonia oxidase does. These are possible candidates for biochemical assay as is the nitrite oxidase of phase II organisms. However, the efficiency of extraction and activity per cell would have to be examined before any use of such techniques could be made.

Srinath et al (1976) define a specific activity coefficient, for nitrification in natural samples, as the ratio of the rate of oxidation (per unit of total nitrogen) in natural systems to that in pure cultures. From this an estimate of the number of nitrifying cells can be calculated. However, the test sample must be under similar conditions, particularly pH and dissolved oxygen, to those of the pure culture. It is not established whether under natural conditions, nitrifying biomass increases proportionally with heterotrophic biomass. In addition the presence of particulate material can grossly affect the chemical environment on a microsite scale. Hall and Murphy (1980) pointed out that comparing pure culture studies with natural samples is hazardous (see also Belser 1979) particularly since interactions between lithotrophic and heterotrophic organisms are disregarded.

## 2.5 Isotopic approaches

Only two isotopes have, as yet, been used in the study of nitrification. These have been the stable isotope Nitrogen-15 ( $^{15}\text{N}$ ) which can be detected by mass spectrometry or directly by  $^{15}\text{N}$  analysis, and the unstable isotope carbon-14 ( $^{14}\text{C}$ ) which can be conveniently detected by liquid scintillation counting. Recently there have been reports of the use of the unstable isotope of Nitrogen ( $^{13}\text{N}$ ) to study nitrogen transformations (Smith et al, 1978) but due to its half life of only 10 minutes complex facilities are required to produce the isotope immediately prior to its use. Schell (1978) is rather optimistic about the future use

of  $^{13}\text{N}$  but if 1 micro Curie ( $\mu\text{Ci}$ , 37 k Bg.) was to be added to the sample, and incubation continued for 1 hour, and the isotope was transported to the laboratory for three hours prior to its use, more than 0.5 Curies (18.5 G Bg) would have to be dispatched and only approximately 0.015  $\mu\text{Ci}$  (0.56 k Bg.) would remain in the sample. Uptake would be considerably less than this. This alone places severe limitations on its general use.

### 2.5.1 Nitrogen-15

These methods utilize the addition of forms of nitrogen, ammonia in the case of nitrification, which are enriched, or sometimes impoverished (Domenach, 1977) in the heavy isotope of nitrogen. This tracer can then be identified by the methods given above after fractionation of the various nitrogen species. Schell (1978) gives a brief review of these techniques and also a full bibliography. The calculation of the rates of transformation using this type of data are given in Brezonik (1968) and Christofi (1978). Although the method seems attractive for nitrification studies there are a number of disadvantages. For mass spectrometry, or  $^{15}\text{N}$  analysis, nitrogen must be in the gaseous form and so each nitrogen species must, after fractionation, be converted to  $\text{N}_2$ . These methods are often time consuming and recoveries often low. Schell (1978) illustrates the problem for inefficient recovery of  $^{15}\text{NH}_3$ . Mass spectrometry is also a relatively insensitive technique, but probably better in terms of precision than available  $^{15}\text{N}$  analysers (Schell, 1978), and large amounts of  $^{15}\text{N}_2$  are usually required. Brezonik (1968) encountered problems of this type, in nitrification studies on Lake Mendota, and often failed to obtain rate data due to the small nitrate fraction. To overcome this problem relatively large concentrations of heavy isotope are added to the sample and this perturbs natural concentrations and therefore sample conditions. Brezonik (1968), for example, added

100  $\mu\text{g l}^{-1}$   $^{15}\text{N}$ , as  $\text{NH}_3$ , whilst natural concentrations rarely exceeded this value in aerobic water; and Miyazaki et al (1975) enriched marine systems with 140  $\mu\text{g l}^{-1}$   $^{15}\text{N}$  whilst the maximum in situ  $\text{NH}_3$  concentration was 14  $\mu\text{g l}^{-1}$  (as N). Schell (1978) states that the current uses of  $^{15}\text{N}$  could be approaching its limits of resolution given as  $10^{-5}$  M (as N) for detection of nitrate, nitrite and ammonia in initially aqueous samples.

Another disadvantage in the use of  $^{15}\text{N}$  is the dynamic nature of the nitrogen cycle in natural samples. If incubation is prolonged there is no control over other retransformations of the  $^{15}\text{N}$ , particularly if it is the dominant isotope. The method might be improved by the incorporation of nitrification inhibitors. Miyazaki et al (1975) used N-Serve to determine the contribution of dissimilatory nitrate reduction to the formation of nitrite maxima observed in marine depth profiles.

The natural  $^{15}\text{N}:^{14}\text{N}$  ratios can vary in different ecosystems, and the various microbial transformations can selectively fractionate the two species. However, this fractionation is likely to be negligible when compared to the precision of mass spectrometer data.

### 2.5.2 Carbon-14

This isotope is used routinely in biology and it offers the sensitivity characteristic of all radiotracer techniques. Its use in the study of nitrification is based upon the addition of  $^{14}\text{C}$  bicarbonate and measurement of its uptake by the autotrophs. Although under discussion at present (Whittenburg and Kelly, 1977) the classical definition of an autotrophic microorganism is one which is able to synthesise all cellular materials for growth from carbon dioxide. It is well established that heterotrophic bacteria can also incorporate carbon dioxide through various anaplerotic biochemical pathways and therefore nitrification inhibitors must be used.

The general method involves the addition of  $^{14}\text{C}$ -bicarbonate (5-25  $\mu\text{Ci}$ , 185-925 k Bq.) to uninhibited and inhibited samples and incubation of these in the dark, in situ (Christofi, 1978), or in the laboratory at in situ temperatures (Billen, 1976). The samples are then processed, counted and the total carbon dioxide uptake, in the presence and absence of inhibitor, calculated (see Materials and Methods). The main objections to the method are i) the specificity of the inhibitor and ii) the reliability of carbon uptake as a measure of nitrifying activity.

In all the studies in which this method has been used (Billen (1976); Indrebø et al (1979); Vanderborght and Billen (1975); Somville, (1978); Christofi (1978)) N-Serve has been the inhibitor. The specificity and characteristics of this have been discussed (2.3.2a). Somville (1978) reported N-Serve sensitive carbon uptake in anoxic environments and attributed this to the activity of sulphate reducing bacteria. Moreover, Jorgenson (1977) noted sulphate reduction in essentially aerobic environments and therefore inaccuracies could occur under these conditions (see for example Indrebø et al (1979)). It is highly likely that N-Serve also inhibits the methane oxidising bacteria and these organisms are also capable of oxidising ammonia to nitrite. Although their contribution to nitrification in aquatic environments is unknown (Rudd and Taylor, 1980) their participation cannot be excluded. O'Neill and Wilkinson (1977) found ammonia oxidation by Methylosinus trichosporium to have similar oxygen to nitrogen stoichiometries as the oxidation by Nitrosomonas and therefore the methods involving measurement of these variables will yield similar results despite the possibility of the involvement of different species. However, there are differences in the biosynthetic pathways of these two organisms and Brock (1977) states that the  $\text{CO}_2$  uptake by methylotrophs is small in relation to that of the autotrophs. Therefore the use of carbon dioxide uptake to determine rates of nitrification may lead to errors.



Also worthy of note here is the use of organic carrier solvents for the N-Serve; although all workers employed suitable controls the effect of the solvent on gross carbon uptake activity has not been investigated and the sample could be changed from in situ conditions.

Billen (1976) converted N-Serve sensitive  $\text{CO}_2$  uptake rates, measured in marine sediments, into nitrogen oxidised values using a factor of 8.3 obtained from published carbon fixed to nitrogen oxidised ratios for the nitrifiers. Christofi (1978) used a factor of 9.7 measured from an enrichment culture whilst Indebro et al (1979) presented the N-Serve sensitive  $\text{CO}_2$  uptake values as a relative index of nitrifying activity. Both Billen (1976) and Christofi (1978) observed good relationships between field nitrate data and estimated nitrification rates.

Finally, Sorokin (1972) described a method, utilizing  $^{14}\text{C}$  which assessed the nitrification potential of a biocenosis. This involved the addition of  $^{14}\text{C}$ , as bicarbonate, to a sample in addition to the specific substrate, ammonia, and any stimulation of carbon uptake noted. As for the potential experiments discussed earlier (2.2.2a) no information is obtained on in situ rates and sample additions (ammonia) remove the sample from in situ conditions.

## 2.6 Mathematical models of nitrification

A full description of all the models proposed is beyond the scope of the present work and the competence of the author. However, in a review of nitrification methodology some attention must be paid to this expanding field. Much work has necessarily concentrated on wastewater treatment facilities which again are not directly relevant to this present review and reference should be made to the brief review by Sharma and Ahlert (1977). Of the natural systems which have been modelled, rivers have received most attention, although Ikeda and Adashi (1976) presented

a series of non-linear differential equations to predict the dynamic behaviour of the nitrogen cycle in lakes. The modelling of dissolved oxygen consumption in estuaries, of which nitrification is an integral component, is undertaken by a number of Water Authorities (for example see Mackay, 1979).

The mathematical bases of many of the river models are the well-known Monod growth equations developed for continuous flow conditions (Monod, 1950) and/or rate equations based on Michaelis-Menten kinetics, relating reaction rate to substrate concentration (Laudelot, 1978). Much of the input data required to operate these models cannot be obtained easily from field studies. The yield coefficient for the nitrifying organisms, for example, has been derived from thermodynamic calculations (Stratton and McCarty, 1967), laboratory studies under field conditions (Remacle and DeLeval, 1977) or from published figures in the literature (Garland, 1973).

The rate equations developed for a number of systems may follow first order kinetics (Garland 1973) where the rate of reaction is proportional to the substrate concentration (i.e.  $V = k [S]$ ), where  $k$  is the first order rate constant; or zero order kinetics (Huang and Hopson, 1974) where the rate of reaction is independent of substrate concentration. Lopez-Bernal et al (1977) presented chemical data from a series of streams and found that in a number of cases substrate disappearance, or product appearance, could be adequately described by either first or zero order kinetics (most studies assume that substrate is the only factor limiting growth).

Wezernak and Gannon (1968) proposed a model based on an autocatalytic growth equation. This type of model requires that the rate of reaction be dependent on the product of the reaction, in this case nitrifying biomass. In other words the reaction is first order with respect to

biomass. Such a model can only be used when growth is occurring with progression downstream and is useful when retention times greatly exceed the generation times of the nitrifying organisms (Estfandi, 1977). In this latter case the appearance of nitrification products would occur at some point downstream of an effluent discharge. Although this is a model of planktonic nitrification it is generally considered that benthic nitrification is the dominant process (Garland, 1976; Finstein et al, 1977, 1978; Gujer, 1977, 1978) i.e. nitrification products appear immediately downstream of an effluent discharge. The basic equations for all the above models are given in Table 2.5.

The reaction rate constants are usually determined by following changes in substrate or product with distance downstream; Lopez-Bernal (1977) stated that this was the best method due to difficulties in determining in situ nitrification rates. There are a number of disadvantages however in carrying out this type of study as discussed in section 2.3.

There are many problems encountered in developing methodology suitable for the study of nitrification and there is much criticism of the application of kinetic data obtained from laboratory cultures to populations in situ. Many of the above models require estimates of nitrifying biomass, activities per cell, yield coefficients and growth rate data. The biomass estimates are usually the products of M.P.N. data and the average weight of a single nitrifier cell; estimates of the former parameter are known to be inaccurate and small changes in the weight of a single cell can cause large errors in biomass estimates (see Ondrako, 1975). Recently Belser and Schmidt (1980) have found differences in generation times, activities per cell and growth yields in different genera and even in strains of the same species of ammonia oxidising bacteria. These differences are all probably related to biomass (cell

Table 2.5 Proposed equations to form the basis of mathematical models for chemolithotrophic nitrification

(from Tiedeman, 1977)

Model	Equation	Comments
Monod	$V = \frac{ds}{dt} = \frac{k S Xc}{K_m + S}$ <p> <math>V</math> = Rate of reaction  <math>S</math> = Substrate conc<sup>n</sup>.  <math>t</math> = time  <math>k</math> = Growth rate const.  <math>Xc</math> = Bacterial conc<sup>n</sup>.  <math>k_m</math> = Half saturation constant                 </p>	Reaction rate dependent upon substrate and bacterial concentrations. If $s \gg k_s$ model approximates a zero order model
Zero order	$V = \frac{ds}{dt} = k_2$ <p><math>k_2</math> = Zero order rate constant</p>	Reaction rate independent of substrate concentration
First order	$V = \frac{ds}{dt} = a s^b$ <p> <math>a</math> = Growth rate constant  <math>b</math> = Exponential order of reaction                 </p>	Reaction rate dependent upon substrate concentration
Autocatalytic	$V = \frac{ds}{dt} = k_3 S (A-S)$ <p> <math>k_3</math> = Reaction rate constant  <math>A</math> = Upper limit of substrate conc<sup>n</sup>.  <math>S</math> = Substrate oxidised                 </p>	Reaction rate dependent upon substrate and bacterial concentrations

size) variations but as it is known that different genera can dominate different ecosystems, in soils at least, (Belser 1979), the problems of interpretation of mathematical models are exacerbated. Such difficulties have reduced a number of studies to simple curve fitting exercises where kinetic parameters are varied until field data are adequately fitted, in other words the model is validated (Laudelot, 1978). Laudelot (1978) also explains that many models attempt to describe too great a level of complexity. A more useful approach would be to construct sub-models which describe part of the system satisfactorily and then build up the complexity by fitting sub-models together. The advantages of this sub-model approach can be appreciated when equations such as those for organisms decay or death (Stratton and McCarty, 1967), and for sediment resuspension and settling (Garland, 1973) are required in the overall model. Other variables which could be included are zooplankton grazing (Ikeda and Adashi, 1976), actual nitrification rates (Cavari, 1977) and reaeration coefficients (presumably related to turbulence) particularly when the model is describing oxygen depletion in rivers.

Bansal (1976) proposed a model based purely on the hydraulic characteristics of river waters and although good relationships were established a number of criticisms were raised (Gujer, 1977; Brosman, 1977) and the author conceded that biological parameters should be included.

Vanderborgh and Billen (1975) produced a diagenetic model to describe the nitrate concentrations in the interstitial water of marine sediments, and Billen (1978) extended this to include ammonia concentrations. In the former model nitrification rates were measured directly, and denitrification was assumed to be first order with respect to nitrate concentrations; an assumption common to many studies but not based on actual measurements (Laudelot, 1978). The assumption in this case was almost certainly valid, but in the second model Billen (1978) estimated the

denitrifying potential from kinetic data based on laboratory experiments.

## 2.7 Summary

In this section some of the difficulties encountered in measuring nitrification have been examined and it is hoped will aid interpretation of results. Despite the apparent possibilities presented by the use of nitrification inhibitors comparatively few quantitative studies have been carried out. This situation is at present changing, due primarily to the recognition of the importance of nitrification in the global nitrogen cycle, an importance which is largely overlooked in many modern textbooks, e.g. Fenchel and Blackburn (1978).

## CHAPTER 3

Review of the literature on nitrification studies in the lacustrine environment.

This chapter reviews the literature on nitrification in the lacustrine environment. Reviews on other aspects of nitrification not directly relevant to the aquatic ecosystem can be found in Focht and Verstraete (1977), Aleem (1977), Belser (1979), Matin (1978), Kelly (1978), Nicholson (1979) and Sharma and Ahlert (1977).

### 3.1 Introduction

Of all the biologically mediated reactions of the nitrogen cycle, the nitrification and ammonification (nitrogen mineralisation) reactions have been the most neglected in studies on the lacustrine environment. This is primarily because these are reactions which involve internal cycling of nitrogen compounds and not sources into, or sinks from, the lake system. These source and sink reactions have been primary centres of attention due to the recognition of the major role that nitrogen plays in the eutrophication of lakes (see Vollenweider 1968).

Over the last 10 to 12 years this situation, particularly with regards to nitrification, has changed, due primarily to the recognition of the possible quantitative significance of the reaction in determining the oxygen demand of water bodies and the production of nitrate for subsequent denitrification. Implicit in this argument is that the lithotrophic nitrifiers are the mediators of the oxidative reactions as these organisms consume large amounts of oxygen relative to nitrogen oxidised and nitrate is the major end product.

### 3.2 General

In his review of the nitrogen cycle in lake waters Hutchinson (1957) stated that the organisms represented by Nitrosomonas and Nitrobacter are the commonest nitrifiers in lake waters but he offered no supporting evidence, even though the occurrence of heterotrophic nitrification, in culture at least, was well established (Meiklejohn 1953). Brezonik (1968) cites Delwiche (1965) stating that the relative importance of heterotrophic and lithotrophic nitrification is as yet unknown. Painter (1970, 1977) concludes that whilst the measured rates of oxidation by heterotrophs are low, some authors think that they may play an important rôle in soil nitrogen metabolism. Keeney (1973), reviewing nitrogen cycling in sediment water systems, quotes soil references and concedes that heterotrophic nitrification could be important in acidic environments. Hutchinson (1957) quotes data showing nitrate to be absent from acid lakes, but this could be due to assimilation being dominant over nitrate production. Verstraete (1976) concludes his review of heterotrophic nitrification by saying that the ecological significance of this process still has to be established, although Keeney et al (1971) states that the trophic state of the lake may determine which organisms are active, with heterotrophs becoming dominant in the highly eutrophic systems. In spite of these doubts some information exists which points to the dominance of the lithotrophic process in aquatic systems. This work has involved the use of the inhibitors discussed in Chapter 2.

Chen et al (1972a) used this approach on Lake Mendota sediment slurries and found that  $1 \text{ mg. l}^{-1}$  N-Serve inhibited the onset of nitrification for three days under aerated conditions. Similar results were obtained for Lake Wingra (Isirimah et al 1976), Loch Balgavies and Blelham Tarn sediments (Christofi 1978). Both lakes Wingra and Balgavies are shallow, eutrophic, normally well mixed lakes, whilst Lake Mendota



and Blelham Tarn are subject to thermal stratification. The sediments therefore would be subject to different thermal regimes, the well mixed lakes showing seasonal cycles and the stratified lakes showing relatively little change in profundal temperatures. It is also worth noting that both Lake Mendota and Blelham Tarn develop completely anoxic hypolimnia and therefore the sediments are also subject to differing oxygen regimes than the shallow well mixed lakes. As N-Serve inhibited nitrification in all these sediment types there are indications that increased temperatures or different redox cycles do not select different processes.

Christofi (1978) also found N-Serve to inhibit nitrate accumulation in isolated water samples taken just above the sediment water interface of Balgavies Loch and from the hypolimnion of Blelham Tarn.

These observations are not in accord with current concepts of lacustrine nitrification as described by many modern textbooks. These books (e.g. Fletcher 1979) describe nitrification as an activity confined to the sediments and requiring temperatures of 10 to 15°C for growth of the organisms. Such apparent conflicts, however, reflect the paucity of information in the literature.

The significance of nitrification in the water column is described by Christofi (1978) who, after demonstrating total lithotrophic involvement, used the oxygen to nitrogen ratio described for this process (Wezernak and Gannon 1968) to calculate that the observed nitrate concentration increases between 7 and 9 metres depth in Blelham Tarn accounted for the total oxygen consumption during this period. Therefore, not only does nitrification produce a substrate for denitrification but it also creates the conditions for this to become the dominant process. Hall et al (1978) performed similar calculations for nitrate increases in the whole hypolimnion of Grasmere (a stratified lake in the English Lake District) and concluded that the process accounts for a minimum of 15% of the total oxygen consumption. There are other reports of nitrate accumulation in

hypolimnetic waters. Brezonik (1968) noted increases between the depths of 7 and 17 metres in Lake Mendota between June and July 1966. The largest increase ( $0.6 \text{ mg N l}^{-1}$ ) occurred between 12 and 15 metres. Assuming this is due to in situ production (for other possible sources see Brezonik (1970), Lee (1970) cited in Keeney (1973)) it can be calculated that the nitrate increase at 12 metres accounted for 72% of the total observed oxygen decrease.

Larsen (1977) also observed hypolimnetic nitrate increases, during June and July, in several eutrophic Danish lakes but the graphical presentation of data does not allow accurate calculation of possible oxygen deficits. Paerl (1975) noted increases in nitrate below 100 metres depth in oligotrophic Lake Tahoe California. These increases were small ( $10 \text{ to } 15 \text{ } \mu\text{g N l}^{-1}$ ) and did not result in any appreciable oxygen depletion.

Although it would be inadvisable to generalise from such limited data the importance of lithotrophic nitrification in the lacustrine environment would agree with the data of Finstein and Matulewich (1977), Cirello (1975), Ehkle (1978), Tiedemann (1977), Garland (1977) and Schornik and Ram (1978), all of whom demonstrated lithotrophic dominance in river sediment water systems. The situation in brackish and marine systems is also becoming more defined through the work of Webb and Wiebe (1975), Vanderborght and Billen (1976), Belser and Mays (1980), and the application of techniques such as those developed by Ward and Perry (1980). In soils of neutral to alkaline pH it is accepted that the lithotrophs are the dominant nitrifiers (Alexander and Clark 1965) and this acceptance is reflected in the search for compounds which specifically inhibit this process (see for example Bundy and Bremner 1973). In acid soils the work of Rennie and Schmidt (1977) is clarifying the situation on phase II populations and perhaps preparation of fluorescent antibodies against other isolates (Walker and Wickramasinghe 1979) will yield information on phase I populations.

Lithotrophic nitrification should occur with a nitrogen lost to nitrogen gained ratio of almost unity (some N is lost due to growth). Of the studies in which hypolimnetic chemical changes are observed and lithotrophic activity demonstrated (Christofi 1978; Hall et al 1978) this ratio is less than unity. This suggests concurrent nitrogen mineralization and agrees with the observations of Brezonik (1968) and Keeney et al (1971) who found ammonification rates to increase with depth and during stratification respectively.

### 3.3 Bacterial counts

#### 3.3.1 Introduction

Without exception all studies involving the enumeration of nitrifying organisms in the lacustrine environment have utilized the most probable number (MPN) technique. A full description of this procedure and subsequent statistical analysis of results is given by Meynall and Meynall (1970).

Unfortunately many of the experimental results described do not use the most precise estimate of the MPN (for example see Niewolak (1970)), but it has been possible to calculate 95% confidence limits for most of the results. Few authors present these limits and in this review the following equation (from Jones, 1979) is used to detect significant changes in nitrifying populations.

$$t = \frac{\log \text{MPN}_1 - \log \text{MPN}_2}{0.55 \sqrt{\frac{\log. a_1}{n_1} + \frac{\log. a_2}{n_2}}}$$

where a = dilution interval used, n = replicates at each dilution,

t = Students 't' distribution value (d.f. = ∞), MPN<sub>1</sub> and MPN<sub>2</sub> are the respective counts.

N.B. if a is equal to 10, 0.55 is replaced by 0.58.

Using the appropriate experimental design at a 't' value of 1.96 it is possible to calculate the minimum difference between log. MPN's for them to be significant at  $P > 0.05$ .

### 3.3.2 Lithotrophic nitrifiers

With the variety of media available, the range of substrate concentrations used (Table 2.2), the variable incubation times and temperatures, plus the inherent difficulties involved in counting these organisms (see page 17-19), comparison between different studies is impossible. However, within a particular study these variables become constant and valid comparisons can be made.

#### 3.3.2(a) Seasonal cycles

Christofi (1978) reports the seasonal cycles of lithotrophic nitrifiers at various depths in the water column of Balgavies Loch between February 1975 and March 1976. Confidence limits were not presented but from the experimental design (1:10 dilution interval, 5 replicates dilution<sup>-1</sup>) a change in log. MPN of 0.72 is required for differences to be significant ( $P > 0.05$ ).

In surface waters there were population increases for both phase I and II organisms from February to March and both remained unchanged throughout April. This coincided with the spring diatom bloom. Phase I counts were 25 bacteria ml<sup>-1</sup> and phase II 6 bacteria ml<sup>-1</sup>. Both populations declined in May and showed no change throughout the summer months. In October both populations increased to maximal values which were sustained until March 1976.

The general pattern of spring and winter population maxima, the latter being the greatest, was also observed at depths of 3 and 8 metres. Numbers in the water column varied from undetectable (less than 1 bacteria ml<sup>-1</sup>) to  $2.5 \times 10^3$  ml<sup>-1</sup>.

The counts for both phase I and II organisms in February 1976 were significantly greater than those for February 1975, heterotrophic counts did not show this variability so it is unlikely to be an inflow effect. Ammonia concentrations and pH increased between these sampling dates by  $500 \mu\text{g N l}^{-1}$  and 1.3 units respectively. Without further data it is not possible to attribute these results to particular controlling factors. Christofi (1978) claims that generally, in Balgavies Loch, the phase I count was greater than the phase II count but this was only true in 24% of all the samples.

Christofi (1978) also describes the populations at 1, 6, 7 and 11 metres in one of the Lund tubes (Lack 1974) in Blelham Tarn between August 1976 and February 1977. At, and below, 6 metres depth there were no changes in the phase I population; phase II populations fluctuated but with no general trends. Many counts were obtained from anoxic water samples which demonstrated the survival of nitrifiers under adverse conditions. There was no indication, at these depths, of the winter maximum observed throughout the water column of Balgavies Loch. In the surface water samples (of the Lund tube) populations were significantly lower than in the deeper waters and these showed no change until overturn when populations were equal throughout the water column. As the counts in the deeper water showed no change from pre-overturn levels growth must have occurred, probably as a result of the mixing of ammonia rich hypolimnetic water. The surface water therefore showed a winter maximum nitrifier population also noted for Balgavies Loch.

The range of observed counts were 1 to  $631 \text{ bacteria ml}^{-1}$  and 1 to  $316 \text{ bacteria ml}^{-1}$  for phase I and II populations respectively and were therefore much lower than the maximal counts observed for Balgavies Loch. Again Christofi claims that phase I counts were significantly greater than those for phase II nitrifiers but significant values were observed in only 6% of the samples.

Niewolak (1970) investigated the seasonal cycles of nitrifier populations at different sites in the sediments of the Iłowa lakes, Poland. The MPN design (1:10 dilution interval, 3 replicates per dilution) indicated that the log. count needed to change by 0.93 for differences to be significant. The MPN estimates were reported as factors of 10 and therefore one log cycle was used as the criterion of significance. There was a general indication of seasonal cycles, with population maxima regularly established in the summer months correlating with the maximum sediment temperature; winter population minima correlate with low temperatures between the months of December and March. Again phase I counts were claimed to be greater than those for phase II but the differences were only significant in 50% of the samples. The numbers ranged from undetectable to  $10^3$  bacteria  $g^{-1}$  wet weight.

Laurent (1971) reports the seasonal cycle of nitrifiers in the sediment of Lake de la Rouille, France, but does not describe the experimental design; without this information no confidence limits can be placed on the results. Phase I numbers ranged between 50 and 1000 bacteria  $g^{-1}$  wet weight and phase II by 15 to 250 bacteria  $g^{-1}$  wet weight.

Christofi (1978) followed the seasonal cycle of nitrifiers in the top 5 cm of sediment in Balgavies Loch. Phase I and II populations increased from April to July concomittant with a temperature increase of 5 to 15°C. At this time the sediments became anoxic and the counts decreased. Oxidic conditions were restored in autumn when population maxima of  $10^5$  bacteria  $g^{-1}$  dry weight were established. The phase II population increase lagged behind that of the phase I organisms by two months but then the two populations remained unchanged till March. On all but three sampling occasions the sediment counts were significantly greater ( $P > 0.05$ ) than the maximum counts observed in the water column. Again claims of phase I predominance over phase II were not supported statistically, the difference being significant on only a few occasions.

Christofi (1978) also studied the population fluctuations in the profundal sediments of tube A in Blelham Tarn between August 1976 and February 1977. The sediments were anoxic in August and both populations decreased until the overturn in October, when counts increased and attained a maximal value in December of  $10^6$  and  $10^5$  bacteria  $g^{-1}$  dry weight for phase I and II organisms respectively. These counts were significantly greater than the maximal counts obtained from the water column on all but one occasion.

### 3.3.2(b) Other observations

Gode and Overbeck (1972) reported that counts of phase I nitrifiers did not exceed 1 cell  $ml^{-1}$  whilst phase II counts reached 200 bacteria  $ml^{-1}$  between April 1967 and June 1969 in the upper hypolimnion of Plubsee, a typical eutrophic stratified Baltic lake. Interestingly the highest counts of the phase II organisms were obtained from anoxic water and sediments.

Landner and Larsson (1973) studied population changes in the Bay of Köpping, Lake Malaren, which resulted from an inflow containing high concentrations of reduced nitrogen. Phase I counts in the inflow varied between 0.2 and 11  $ml^{-1}$  but the count at the first station in the lake (samples taken from just above the sediments) was  $10^4$  bacteria  $ml^{-1}$  indicating rapid growth had occurred. The numbers then declined to less than 2 bacteria  $ml^{-1}$  at the station furthest into the lake. Ammonia, nitrite and nitrate all decreased in concentration with distance into the lake. These decreases were probably caused by dilution effects. Phase II populations never exceeded 2 bacteria  $ml^{-1}$ . As part of the same survey these authors noted population changes in the sediment. Numbers of phase II organisms varied between 10 and  $3 \times 10^3$  bacteria  $g^{-1}$  wet weight (counts of  $10.5 g^{-1}$  wet weight are significantly greater than the water column populations). Numbers of ammonia oxidisers were not

presented but the ratio of phase I to phase II bacteria ranged from 10 to  $10^3$  indicating a great predominance of the former organisms.

The counts of nitrifiers in all the above studies are summarized in Table 3.1

### 3.3.3 Heterotrophic nitrifiers

To account for the observed nitrate increases in the upper hypolimnion of Plubsee and the low counts of phase I nitrifiers Gode and Overbeck (1972) obtained counts of heterotrophic nitrifiers of  $10^4$  bacteria<sup>-1</sup> using an acetate-ammonia broth. These were identified as representatives of Flavobacterium, Xanthomonas, Achromobacter, Arthrobacter, Micrococcus, Pseudomonas and Vibrio, the latter two being the dominant genera. An Arthrobacter isolate was investigated in more detail (Gode and Overbeck 1972, Witzel and Overbeck 1979) and nitrite was found to be the dominant product. Nitrite production was related to the initial acetate concentration of the medium and required magnesium ions. Glucose, tryptone and yeast extract inhibited nitrite production but not growth. Nitrite was only present in trace quantities in the lake but with counts of phase II nitrifiers of 200 cells ml<sup>-1</sup> one could postulate a heterotrophic-lithotrophic interaction producing nitrate. Equally possible, however, is the presence of a dominant heterotroph which produced nitrate directly.

Recently Christofi (1978) has criticised these results based on his observations that regular additions of N-Serve (1 mg l<sup>-1</sup>) to the acetate ammonia broth reduced the heterotrophic nitrifier titre, in samples from Loch Balgavies, by at least an order of magnitude. This indicates that the medium supports the growth of phase I nitrifiers which interfere with the estimated heterotrophic count. However, Christofi used the same mineral salts medium as Gode and Overbeck to count up to  $10^3$  cells ml<sup>-1</sup> in Loch Balgavies water samples. As the incubation conditions of the two workers were almost identical (Christofi incubated for 2 days longer)



Table 3.1. The range of counts for both phase I and II chemolithotrophic nitrifying organisms in the water column and sediments of various lakes.

Lake	Water Phase		Sediments		Reference
	Phase I ( $\text{ml}^{-1}$ )	Phase II ( $\text{ml}^{-1}$ )	Phase I	Phase II	
Balgavies	$0 \rightarrow 2.5 \times 10^3$	$0 \rightarrow 2.5 \times 10^3$	$3.2 \times 10^3 \rightarrow 3.2 \times 10^{5*}$	$3.98 \times 10^2 \rightarrow 3.2 \times 10^{5*}$	Christofi (1978)
Bielham	$1 \rightarrow 6.3 \times 10^2$	$1 \rightarrow 3.16 \times 10^2$	$3.98 \times 10^2 \rightarrow 10^{6*}$	$2.0 \times 10^3 \rightarrow 10^{5*}$	
Ilowa Lakes			$0 \rightarrow 10^3$	$0 \rightarrow 10^3$	Niewolak (1970)
Rouille			$50 \rightarrow 10^3$	$15 \rightarrow 2.5 \times 10^2$	Laurent (1971)
Plubsee	$0 \rightarrow 1.0$	$0 \rightarrow 2 \times 10^2$			Gode & Overbeck (1972)
Malaren	$2 \rightarrow 10^4$	$0 \rightarrow 2$	$10^2 \rightarrow 3 \times 10^4$ (b)	$10 \rightarrow 3 \times 10^3$	Landner & Larsson (1973)
			$10^3 \rightarrow 3 \times 10^6$ (b)		

\*  $\text{g}^{-1}$  dry wt. all other values  $\text{g}^{-1}$  wet weight. b. calculated from reported Phase I : Phase II ratios.

it is impossible, without performing the same tests on the samples, to speculate on the reasons why these organisms were not detected, if they were present, in the Plubsee samples.

Christofi (1978) followed the seasonal variations in heterotrophic nitrifier counts, using the acetate ammonia broth, in Loch Balgavies and Blelham Tarn. It was during this study that he observed that maintaining a concentration of  $1 \text{ mg l}^{-1}$  N-Serve reduced the heterotrophic nitrifier titre by at least an order of magnitude.

In the absence of the inhibitor heterotrophic nitrifier titres reached  $10^2$  bacteria  $\text{ml}^{-1}$  and, in the water column, showed population maxima between January and April. As pointed out by Christofi (1978) the reduction in count caused by N-Serve indicates that the reported numbers in heterotrophic nitrifier populations should be interpreted with caution. The efficiency of N-Serve, to inhibit phase I populations, decreases with increasing organic carbon concentrations (Goring 1962; Bundy and Bremner 1973). Although the organic carbon in the medium may reduce the rate of N-Serve hydrolysis (Redemann et al 1964) it may also reduce the inhibitory effect of  $1 \text{ mg l}^{-1}$  N-Serve; therefore the activity still detected could be due to lithotrophic nitrifiers. These organisms are known to assimilate organic carbon monomers (for review see Matin, 1978) and their ability to nitrify in heterotrophic environments is well established. The only other report on the isolation of a heterotrophic nitrifier from lake samples was by Laurent (1971) who demonstrated both lithotrophic and heterotrophic nitrifiers in Lake de la Rouille sediments. The heterotrophs were dominated by Arthrobacter species and nitrification in culture was inhibited below  $12^\circ\text{C}$ . Interestingly he claimed that both lithotrophs and heterotrophs nitrify under aerobic conditions but heterotrophic activity persisted to a redox potential of  $-85 \text{ mV}$ , although this is not a priori evidence for the absence of oxygen (dominant redox couples could poise the Eh at this level).

Sterilized sediments were rapidly recolonised by the Arthrobacter isolates and nitrate was produced suggesting that the organism was different to the isolate obtained by Gode and Overbeck (1972) and Witzel and Overbeck (1979).

Isirimah et al (1976) concluded that heterotrophs were responsible for the nitrite accumulation in stored Lake Wingra water samples as lithotrophs could not be detected by MPN analysis.

#### 3.4 The relationship between nitrifying activity and population estimates.

As counts of nitrifiers in soils have underestimated population levels (Rennie and Schmidt 1977) and difficulties have been encountered in counting these organisms in freshwater environments (Finstein and Bitzky (1972); Matulewich et al (1975)) it would seem opportune to review how the counts relate to activity measured by other methods.

Christofi (1978) detected both phase I and II populations at all sampling depths throughout the year in Balgavies Loch. However, using the potential technique (in situ temperature with no ammonia supplement), activity was only detected in September at three and eight metres depth. Phase I populations at this time were not significantly different from the populations in either August or October. Phase II populations increased in September at the 3 metre depth. Nitrate was the dominant product at 3 metres but nitrite was dominant at 8 metres where phase II populations were smaller. Using the same technique, sediment and water samples taken in January were incubated at room temperature. Increases in nitrate concentration in the sediments were two orders of magnitude greater than those observed in water samples. This reflected the larger populations found in the sediment (see Table 3.1).

The increases in nitrate concentration between 7 and 9 metres in Blelham Tarn during August were not accompanied by changes in either

phase I or II populations but the N-Serve sensitive bicarbonate uptake (see page 49 ) did increase, and using a conversion factor of 8.3 a value of  $45 \mu\text{g N oxidised l}^{-1} \text{ day}^{-1}$  was obtained. The rate from observed nitrate increases was less, at  $33 \mu\text{g N l}^{-1} \text{ day}^{-1}$ , probably due to in situ nitrate utilization. This illustrates that population densities may not signify changes in actual rates of activity.

Christofi (1978) also noted a peak in nitrate concentration preceded by a nitrite peak, in the sediments of Blelham Tarn at the end of September 1976, but populations of both phase I and II organisms again showed no significant increases. A further peak was noted in December which did correlate with greatly increased populations of nitrifying organisms (but also with the minimum recorded activity of nitrate reduction). The maximum recorded nitrification potential, measured in October, was accompanied by population minima of both phase I and II organisms. An accumulation of nitrate was observed in the sediment interstitial water of Loch Balgavies from April to July; an increase in phase I but not phase II numbers was also noted. The nitrification potential of the sediments increased during this period and was positively correlated with temperature increases. Upon development of anoxic conditions counts did decrease but high numbers were still present. The nitrification potential of undisturbed sediments at this time was zero but activity could be induced by aeration.

Using the maximum counts obtained and culture yield estimates Christofi (1978) could not account for any of the observed nitrate increases in water or sediment samples from Loch Balgavies. The use of culture yields in field situations has been discussed by Belser (1979) and in situ yields are considered to be substantially less indicating that Loch Balgavies populations are grossly underestimated.

Niewolak (1970) observed that nitrification potentials followed the seasonal temperature cycles, being greater during the summer periods and

coinciding with nitrifier population maxima. All incubations were at 25°C and so the results reflect population levels present in the sample and not temperature effects on rates of activity. However the potential oxidation rate for the nitrite oxidisers was approximately four times that measured for ammonia oxidisers, a difference which is not reflected in population estimates. Landner and Larsson (1973) found nitrite to be the major end product of nitrification potential experiments on Lake Malaren water in which phase I populations exceeded phase II organisms by a factor of 5 to 5,000. However, sediment samples produced only nitrate whilst reported ratios of phase I to phase II organisms gave values of 10 to 1000.

With the exception of the few data presented by Christofi (1978) on independently measured nitrification rates, most workers have attempted to associate counts with the nitrification potential of incubated samples. Whilst this approach has serious limitations (e.g. due to nitrate and nitrite utilization by other bacteria active in the sample, O'Neill and Holding 1975) it provides some indication that the accumulation of nitrification products is due to the growth and activity of the nitrifying bacteria present in the sample. Although these organisms must increase in numbers during the incubation period (2 to 28 days) the activity measured is assumed (if incubation conditions are standard) to relate to the initial populations present within the sample. The above results show this is very rarely the case.

It has been well documented (Alexander 1965, Belser 1977, Landner and Larsson 1973) that for oxidation of a fixed amount of substrate phase I should outnumber phase II organisms. From this review it becomes apparent that this is not so although there are no data on the relative efficiencies of media used for counting phase I and II organisms. Matulewich et al (1975) have recorded incubation times of 20 to 55 days and 103 to 113 days respectively for counts of phase I and II organisms

to reach maximal values. Although the media used in this study differ from those used in lacustrine studies the incubation times used for the latter (up to 30 days) could result in underestimates of the phase II titre. Belser (1977) reports that denitrification can be an important source of nitrite for phase II populations and this would result in preferential growth over phase I populations. The significance of this reaction in lacustrine samples is unknown.

### 3.5 Rates of activity

The reported rates of nitrifying activity, measured by various methods, in water and sediments of different lakes are summarised in Tables 3.2 and 3.3. The only generalization that the author wishes to make here is that the nitrification potential of sediments is much greater than that of the water column when measured under similar conditions. Even this is made with caution due to the methods used to obtain the sediment slurry for these experiments. Isirimah et al (1976) and Chen et al (1972a) diluted the sediment by 50% using deionized water, Christofi (1978) prepared the same dilution using filter sterilized water overlying the sediment whilst Landner and Larsson (1973) inoculated the sediment into a medium supplemented with ammonia. The results of Landner and Larsson (1973) are not presented in Table 3.2 as the results were expressed as per cent nitrogen oxidised and the initial concentration was not reported.

The high values reported by Seppanen and Wunderlach (1970), for surface waters of Hiidenvesi, Finland, are probably a result of the method used where the water was inoculated into an ammonia containing medium.

Another very general observation is that with the exception of Cox Hollow Lake, where all rates were very low, nitrifying activity in surface waters is either the same, or considerably less, than that observed for deeper waters. Brezonik (1968) noted that although nitrification occurred in the surface waters of Lake Mendota the rate of nitrate supply could

Table 3.2. Nitrification rates, estimated by different methods, in the water column of various lakes.

Method	References	Lake	Date	Depth (m)	Rate $\mu\text{g N l}^{-1} \text{ day}^{-1}$	Comments	
$^{15}\text{N}$	Brezonik (1968)	Mendota	March 1966	1	5.0	Many other attempts to estimate nitrification rates were made but were unsuccessful due to the sensitivity of $^{15}\text{N}$ analysis and the small nitrate fraction.	
				7	9.6		
				15	9.6		
			April 1966	1	8.6		
				7	8.6		
			May 1966	15	8.4		
			June 1966	1	7.0		
		20	6.7				
Nitrification potential	Larsen (1977)	Hald	-	-	7.0	This was the highest rate measurement.	
	Christofi (1968)	Balgavies	Sept. 1975	3.0	1.8 ( $\text{NO}_2$ ) 2.85 ( $\text{NO}_3$ )	Measured separately on ammonia or nitrite enriched lakewater incubated at <u>in situ</u> temperatures.	
				8.0	27 ( $\text{NO}_2$ ) 8.6 ( $\text{NO}_3$ )		
				January 1976	8.0	29.5 ( $\text{NO}_3$ )	As above but at room temp
	Sepanen and Wunderluch (1970)	Hildenvesi	-	Surface waters		6250	Nitrite production from ammonia in a defined mineral salts medium. Different sample sites used.
					4546		
					zero 3846		

Table 3.2 continued

Method	References	Lake	Date	Depth (m)	Rate $\mu\text{g N l}^{-1} \text{ day}^{-1}$	Comments	
$^{14}\text{C}$ -bicarbonate and N-Serve	Christofi (1978)	Blelham Tarn	August 1976	0	zero )	Mean of two values	
				6	4 )		
				8	35 )		
				11	zero )		
			Sept. 1976	0	zero )		
				6	6.25 )		
				8	17.5 )		
				10	15.3 )		
				11	5.0 )		
				12	zero )		
Temporal changes in nitrate concentrations	Brezonik (1968)	Mendota	June/July 1966	12	17.6		
			Cox Hollow	March/July 1966	Surface*	0.9	
				Bottom*	0.7		
				August 1966	Bottom*	0.07	
				August/Sept. 1966	Surface*	0.65	
				Bottom*	0.50		
Christofi (1978)	Blelham Tarn	August 1976	8	42.5			
		Hall et al (1978)	June/July 1975	10-15m	7.0		
			15-20m	12.0			
			June/July 1976	10-15m	8.0		
			15-20m	8.0			
			June/July 1977	10-15m	3.0		
			15-20m	4.0			
		Heaney (unpublished)	Esthwaite	June/July 1972	8	4.0	
				10	7.0		

\* Reference to depth in water column



Table 3.3. Nitrification potential in the sediments of various lakes.

Reference	Lake	Date	Depth into sediments	Rate $\mu\text{g N day}^{-1} \text{ g.wet wt}^{-1}$	Comments
Niewolak (1970)	Ilawa Lakes	July 1962	Undefined	460	Nitrite production from ammonia in a defined mineral salts medium, incubation at 25°C.
		December 1962 to March 1963	"	14 (mean value)	
Chen et al (1972)	Mendota	August 1962	"	1508	Nitrate production from nitrite in a defined mineral salts medium, incubation at 25°C.
	Wingra	December 1962 to March 1963	"	486 (mean)	
	Geneva	-	"	2.53	
	Mendota	-	"	1.79	
Isirimah et al (1976)	Wingra	-	0 - 10 cm	9.7	Assuming 1 ml of sediment weighs 1 g. Sediment diluted 0.5 with deionized water. Incubation at 10°C and stirring. Nitrate production from ammonia. As above but incubation at 25°C.
	Balgavies	1975	0 - 5 cm	8.0	
Christofi (1978)	Balgavies	1975	0 - 5 cm	8.0	Average value over whole year. Nitrate production from ammonia in sediment water slurry. Assume 1 ml sediment weighs 1 g.
	Blelham (Tube A)	1976	0 - 5 cm	2.03	As above

not meet assimilation demands and the nitrate concentration remained very low.

### 3.6 Factors affecting activity

#### 3.6.1 Temperature

Christofi (1978) and Chen et al (1972a) observed that temperature has a great influence on the rate of nitrification. Increasing the temperature of incubation from 10 to 15°C almost doubled the nitrification potential of Lake Mendota sediments whilst increases in the temperatures used to incubate Balgavies sediment (in situ to room temperatures) increased the potential from undetectable to over 4 mg N l<sup>-1</sup> day<sup>-1</sup>. A similar effect was found for Loch Balgavies water samples. These temperature effects have already been noted for shallow lake sediments, and it has also been demonstrated recently (Jones and Simon, in press) that the littoral sediments of Blelham Tarn produce more nitrate than profundal sediments when incubated at similar temperatures; this must be due to a larger nitrifying population in the sediments at the higher temperatures. The inability to detect nitrification in the sediments of Balgavies Loch between December and March, even though nitrifying populations were present, indicates a possible temperature limitation.

The accumulation of nitrate in hypolimnetic water due to the activity of lithotrophic bacteria (Christofi 1978) shows that they can have substantial effects on water chemistry at low temperatures. Larsen (1977) using <sup>15</sup>N techniques demonstrated activity at 6.5°C in Danish lakes.

#### 3.6.2 Oxygen

The strict aerobic nature of lithotrophic nitrification means that the process cannot occur in anoxic water or sediments. However, the organisms can survive in the absence of oxygen and if oxygen is introduced during sampling, or the experiments are carried out under aerobic conditions, nitrification is observed. This latter effect was demonstrated

by Chen et al (1972a), Isirimah et al (1976) and Christofi (1978) when only stirred sediment systems accumulated nitrate; sediments left quiescent showed either no change, or a decrease, in nitrate. Often in these quiescent systems the total ammonia concentration decreased indicating concurrent nitrification and denitrification.

The survival of nitrifiers under anoxic conditions was also demonstrated by Graetz et al (1973) who maintained sediment water systems under anaerobic conditions for 43 days. Reintroduction of oxygen caused the ammonia in the water column to be rapidly and completely oxidised to nitrate. Oxygen limitation can also occur in lake sediments where the oxidised layer rarely extends more than 2 cms into the sediment. Of the work reviewed here the depth of sediment studied varied from undefined (samples taken with an Eckman grab) Chen et al (1972a), Niewolak (1970), to the top ten cms layer (Isirimah et al 1976, Christofi 1978). This limitation is acknowledged by most authors and the effect of stirring on nitrate production 'in situ' will only be reproduced in shallow water sediments subject to turbulence.

### 3.6.3. pH

Chen et al (1972a) found nitrate accumulation in stirred sediments of hard water lakes but not in soft water lake sediment. The hard water sediments contained up to 50% calcium carbonate. This compound, in its soluble form, is known to be an excellent buffer in the neutral to slightly acidic pH range (Stumm and Morgan, 1970), and as nitrification produces hydrogen ions (Bhandari and Nicholas, 1979), the increased activity in calcareous sediments could be due to this buffering capacity. Acid lake sediments amended with calcareous sediments and 5 to 50% calcium carbonate (sediment dry wt. basis) showed rapid nitrate accumulation. It is not clear if this is a direct pH effect as other additions used to reproduce comparable pH levels did not result in nitrate accumulation.

The authors explain this from the high ionic concentrations produced by the latter additives inhibiting any nitrifying activity. The inability of acid sediments to nitrify is unusual as the pH of those studied (5.6 to 6.8) is greater than the pH level of soils known to contain lithotrophic nitrifiers (Rennie and Schmidt 1977; Walker and Wickramasinghe, 1979). These soils may, however, contain buffer systems to maintain a relatively constant pH, whilst the acid sediments were shown to have little natural buffering capacity. Amendments to nitrifier enriched acid sediments which resulted in pH increases to above 9.0 (5% Mg CO<sub>3</sub>, 2% CaO) resulted in nitrite accumulation.

#### 3.6.4 Ammonia concentration

Ammonia is the substrate for nitrification and therefore must be present for activity, however, excessive concentrations can affect this process. A stimulation of nitrite production was noted by Oertli (1972) when ammonia supplemented water from Lake Como was maintained at high pH. The most rapid nitrifying activity was noted between pH 8 to 9 and nitrate was only produced when the ammonia was exhausted. High ammonia concentrations completely inhibited nitrite oxidation and varying degrees of inhibition could be correlated with the free ammonia concentration. Similar results have been reported by Kholebarin and Oertli (1977a) and Anthonisen et al (1976) and it is now well established that free ammonia inhibits phase II nitrifiers. This effect could explain the results of Landner and Larsson (1973) discussed on page 64; however, pH values were not reported.

#### 3.6.5 Light

Rho and Gunner (1978) noted a typical nitrification pattern when water milfoil (Myriophyllum heterophyllum) was allowed to decompose under aerated conditions in the dark. The water was not inoculated so nitrifiers must have been part of the natural epiphytic bacterial population. If the

systems were illuminated the formation of the nitrite peak was delayed for 80 days. Hooper and Terry (1974) have reported photo inactivation of Nitrosomonas by wavelengths of approximately 400 nm. The organisms were most sensitive when oxidation rates were low but reduced oxygen tensions lessened the effect. In surface waters oxygen is often supersaturated due to photosynthetic activity and ammonia concentrations are low due to assimilation. Nitrosomonas could therefore be very susceptible to photoinhibition under the<sup>sa</sup> conditions and this may partly explain the low nitrifying activity detected. The target enzyme appeared to be ammonia oxidase, and as this catalyses the first step in the oxidative energy yielding reaction other phase I genera could be susceptible. Chlorophyll is known to filter out the blue components of light (Odum 1971) and as phytoplankton biomass varies throughout the season so will the depth to which photoinactivation can occur.

### 3.6.6 Suspended particulate material

Kholebarin and Oertli (1977) investigated the effect of suspended particles on nitrification in surface waters. Samples containing sterilized particulate material showed a 60% increase in nitrite oxidation rate over samples with most particulates removed (centrifugation, 1000 x G for 15 minutes). As the supplemented nitrite would not be absorbed to surfaces it was thought that the increased rate was due to the stimulation of phase II organisms by adherence to particles. However, the removal of particles would also reduce the total bacterial population present. Ammonia oxidation showed a 30% increase in rate in the presence of particles but this could be due either to substrate concentration by adsorption, bacterial attachment to available surfaces increasing activity or reduced populations in the centrifuged sample. Using size fractionated particles these authors found that the rate of ammonia oxidation was more closely related to the weight of particulate material present than the available

surface area. As ammonia adsorption would be related to surface area the stimulatory effect could possibly be due to bacterial adherence. This contradicted the findings of Goldberg and Gainey (1955) who, using minerals with varying cation exchange properties, found that surfaces did not stimulate the growth of nitrifiers but that the rate of activity was somehow directly related to the amount of unadsorbed ammonia ions (ie. the cation binding potential).

The increased activity of bacteria in the presence of surfaces is a well known phenomenon although the underlying mechanism is not fully understood. Fletcher (1979) reviews the attachment of bacteria to surfaces in the aquatic environment.

The association and increased activity of nitrifiers in the presence of particulate material in lakes has yet to be established. However, it could explain the greater activity noted in sediments which can be regarded as water containing a high concentration of particulate material (W. Davison pers. comm.). These deposits however, also contain higher nutrient concentrations and consequently greater bacterial populations. Paerl (1975) speculated that nitrification could be associated with sedimenting seston as nitrate was accumulating in water containing undetectable levels of ammonia and nitrite. Seppanen and Wanderluch (1970) found the greatest nitrification potential in waters with high turbidity.

### 3.7 Summary

The many instances where bacterial counts cannot be related to nitrifying activity suggests that the cultural techniques in use at present are failing to cultivate a proportion of the nitrifier population. Johnson and Sieburth (1976) point out that we may be unable to culture the ecologically important organisms. The underestimation of nitrifier populations is not unknown (Rennie and Schmidt, 1977; Carlucci and Strickland, 1968) and clearly there

is a need for research into the culture and isolation of these organisms from natural environments. The characterization of nitrifying organisms from soil is well established (Walker and Wickramasinghe, 1979; Belser and Schmidt, 1978, 1980; Josserand and Cheyet-Marel, 1979; Rennie, 1978) and reports on the marine environment have appeared (Ward and Perry, 1980). Investigations into the lake environment using similar techniques could not only reveal information on levels of counting efficiency of the cultural procedures but also whether specific lacustrine species exist (cf. Nitrosococcus oceanus in the marine environment). It is possible that stimulus for this type of research has been lacking due to the absence of quantitative data to assess the importance of nitrification in lakes. The in situ rate measurements reported are few and there is no information on the seasonal variation. There are no in situ rate measurements at all for sediment systems which, as indicated, are probably the most important site for ammonia oxidation. Of the data reported for sediments little attention has been attributed to the controlling effect of steep redox gradients within the top few centimetres of the deposits and the subsequent fate of the nitrate produced. Work of this type is already available for marine (Vanderborght and Billen, 1975) and soil systems (Phillips and Todd, 1978).

The present work was intended to provide nitrification rate measurements in both water and sediments and to estimate their seasonal variations. It was also an opportunity to investigate the spatial distribution of activity within the sediments and the way this was controlled by differences in redox potential. It was also intended that the use of nitrification inhibitors would provide additional information on the types of organisms which are dominant in an actively nitrifying system.

## CHAPTER 4

## Materials and Methods

## 4.1 Sampling sites.

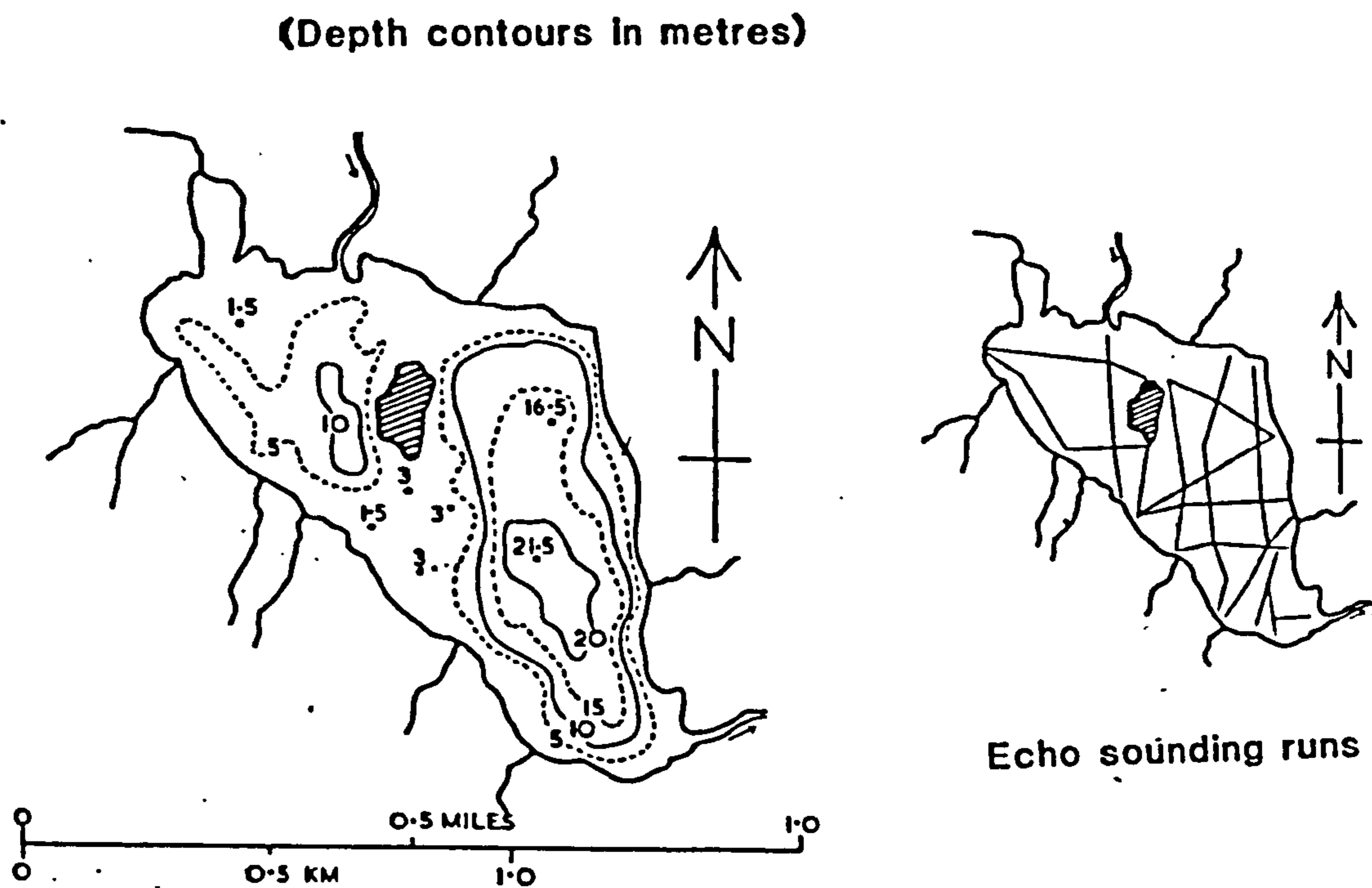
Throughout the course of this work five lakes were sampled, however the major study was conducted on Grasmere and therefore a more detailed description of this lake is provided.

Situated 62 metres above sea level Grasmere (O.S. 065 338) is the northernmost of a series of three lakes in the Windermere catchment basin draining to the south Cumbria coast. It is relatively small being approximately 1.6 km in length and 0.9 km at its widest point. The underlying geology comprises Borrowdale volcanic rocks, the basic geochemistry of which is described by Millward et al (1978). The bathymetric map of Grasmere and specific hydrographic data are presented in Figure 4.1 (from Ramsbottom, 1976). The lake consists of two basins, a small one to the west of the island and the main basin occupying the eastern part of the lake. The lake margins are fringed with Phragmites communis Trin. particularly on the northern shoreline. Littoral vegetation gives way to uncolonised rocky shores to the southern end of the lake, which is also the only region where woodland adjoins the shore. Extensive shallows are to be found around the inflow and to the north east and south of the island. The direction of inflowing water over these shallows is largely governed by wind speed and direction. The morphometry of the lake is such that the volume of hypolimnion is small (24% of the total volume of the lake).

The area of the drainage basin is approximately 28 km<sup>2</sup>, the main inflow being the River Rothay which enters from the north. The land use in this catchment is mainly upland grazing, or pasture, for sheep and beef cattle. The human population is estimated to be around 1000 most of which is concentrated in Grasmere village itself, but increases of up to 300% can occur during the tourist season. The sewage and drainage system of this village, before 1971, comprised septic tanks which emptied by



Fig. 4.1 Bathymetric map and hydrographic data of Grasmere in the English Lake District.



### Hydrographic data

Depth of contour m	Area enclosed by contour		Layer m	Volume of layer	
	km <sup>2</sup>	%		m <sup>3</sup> × 10 <sup>6</sup>	%
0	0.644	100	0-5	2.440	49
5	0.332	52	5-10	1.353	27
10	0.209	32	10-15	0.808	16
15	0.114	18	15-20	0.363	7
20	0.031	5	20-21.5	0.023	0.5
21.5 (bottom)					

Max. depth: 21.5 m    Mean depth: 7.7 m    Total Volume: 5.0 m<sup>3</sup> × 10<sup>6</sup>

seepage and probably resulted in little enrichment of the lake. This led Pearsall and Pennington (1973) to classify the lake as having an oligotrophic plankton, but exhibiting a degree of deoxygenation of the hypolimnion due to its small relative volume (morphometric eutrophication). In the summer of 1971 the sewage system of the village was converted to a small activated sludge treatment plant with effluent discharge into the lakes main inflow, the River Rothay. Unfortunately Grasmere received little attention in the early comparative studies of the lakes in this area and therefore few comparisons can be made to test the effect of the sewage effluent on the lake's nutrient status. Such comparisons as are possible are presented by Hall et al (1978). More recent lake classification schemes presented (for example see Jones et al (1979)) place Grasmere at the eutrophic end of the scale.

For comparative studies four other lakes were sampled. Buttermere (O.S. 160 180) is an oligotrophic lake situated on the Skiddaw slates, the oldest rocks exposed in the Cumbrian mountains (Jackson 1978). This lake often has a greenish colouration due to fine particle suspension from the nearby slate quarries (Cooper 1966). It is approximately 2 km in length and has maximum and mean depths of 28.5 and 16.5 metres respectively. Derwentwater (O.S. 210 260) is a mesotrophic lake situated on the junction of the Borrowdale volcanic rocks and Skiddaw slates. Its length is approximately 3 km with maximum and mean depths of 22 and 5.5 metres respectively.

Windermere (O.S. 986 395), the largest lake in the area, is best treated for our purposes as two lakes, the north and south basins being divided by the shallow areas around Belle Isle. The north basin was sampled during this study and is usually classified as meso- to oligotrophic and has maximum and mean depths of 64 and 25 metres respectively. The underlying geology is described as Bannisdale slates and Coniston grits but to the north a series of different outcrops impinge on the

lake before the Borrowdale volcanic rocks become dominant.

The final lake sampled was Brotherswater (O.S. 128 403) which like Grasmere is situated on the Borrowdale volcanic series. It should be noted that these series of geological formations consist of a variety of different rock types. No recent general survey is available although the rock types for the Brotherswater catchment are described by Chambers (1978). Its length is 0.7 km and breadth 0.6 km with maximum and mean depths of approximately 15.5 and 8 metres respectively.

The bathymetric charts and available hydrological data for these lakes are presented in Figure 4.2 (from Ramsbottom 1976 and Chambers 1978).

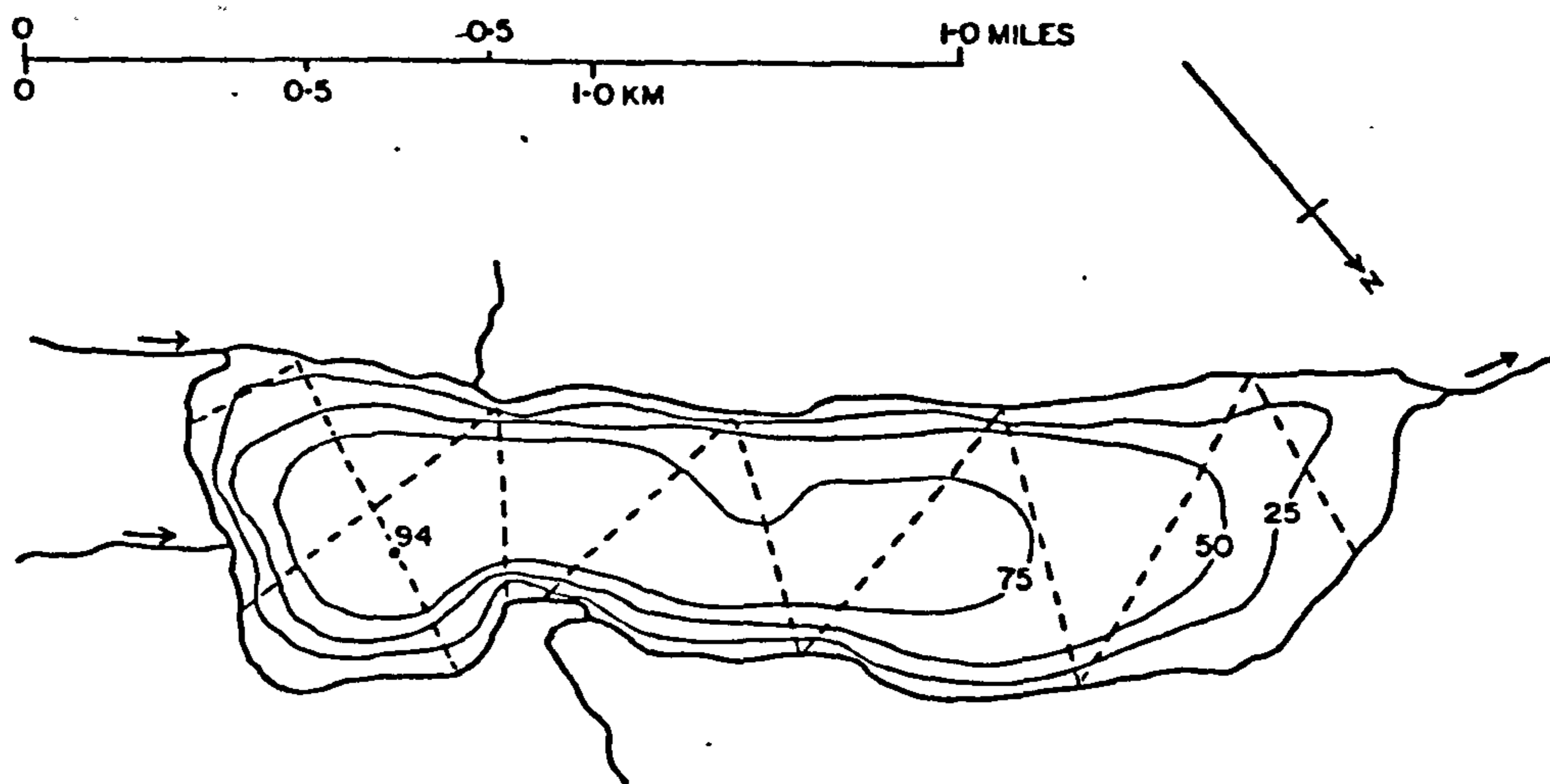
#### 4.2 Sampling points

All the lakes were sampled at their deepest points, however, a number of other sites were investigated in Grasmere and are illustrated in Figure 4.3. Site A was the deepest point of the lake at approximately 21.5 metres. Sites B, C and D were 16.5, 10 and 5 metres deep respectively and sites E and E<sub>1</sub> approximately 0.5 metres deep. The sediments at sites A, B, C and D were typical organic deposits with a light brown oxidised layer extending up to 1.5 cm into the sediment depending upon the actual site and time of the year. The sediments at sites E and E<sub>1</sub> were sandy in nature with a layer of underlying clay.

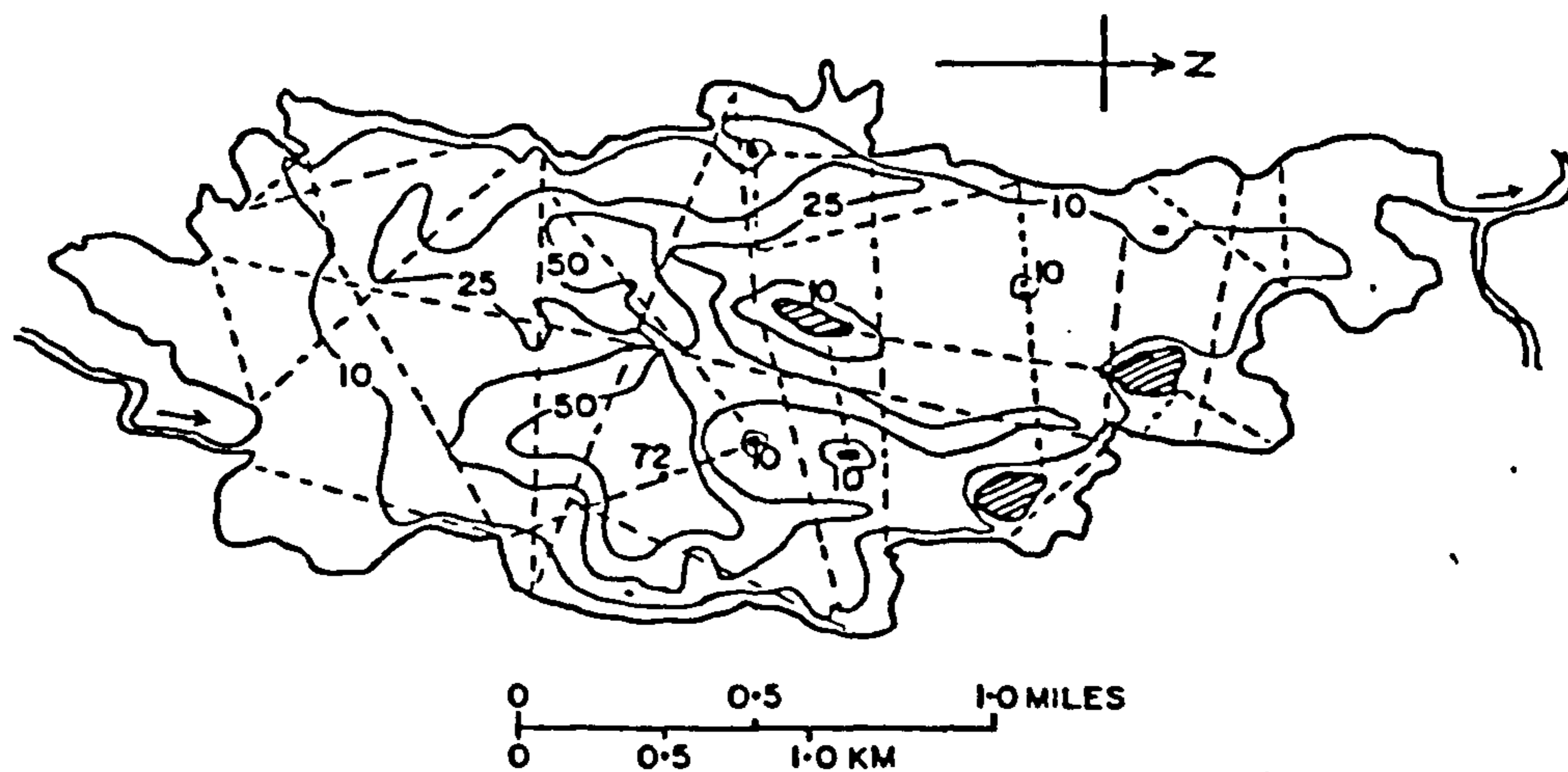
#### 4.3 Location of the sampling point

Sites A, B, C and D on Grasmere, and the deepest point in the north basin of Windermere, were marked by permanently positioned buoys and therefore samples were taken at approximately the same position on each visit; the exact position depended on wind direction. Grasmere sites E and E<sub>1</sub> were not marked but were sufficiently close to the shoreline to take accurate bearings. The positions of maximum depths in Buttermere,

Fig. 4.2 Bathymetric maps of other lakes sampled during the investigation.



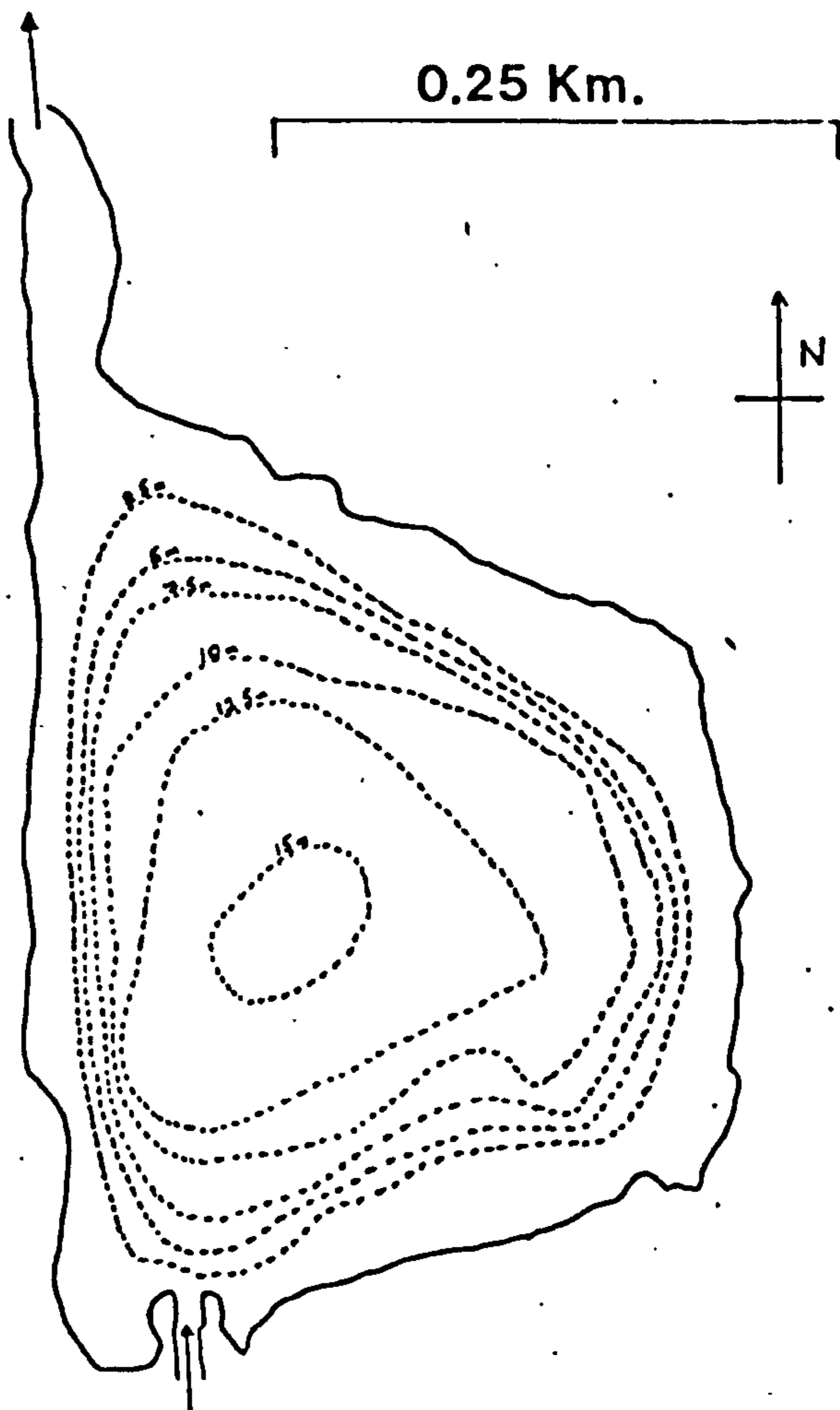
**BUTTERMERE** (Depth contours in feet)



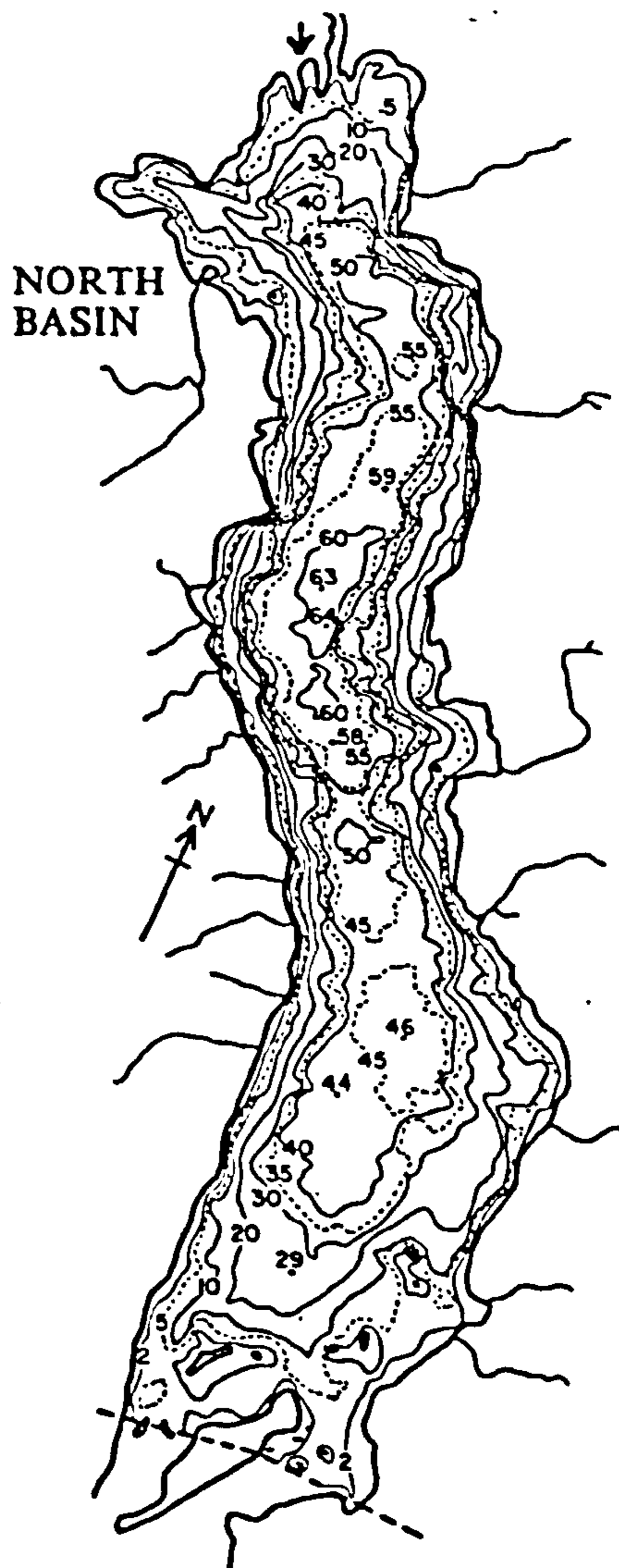
**DERWENTWATER** (Depth contours in feet)

Fig. 4.2 continued.

# BROTHERSWATER



# WINDERMERE



(Depth contours in metres)

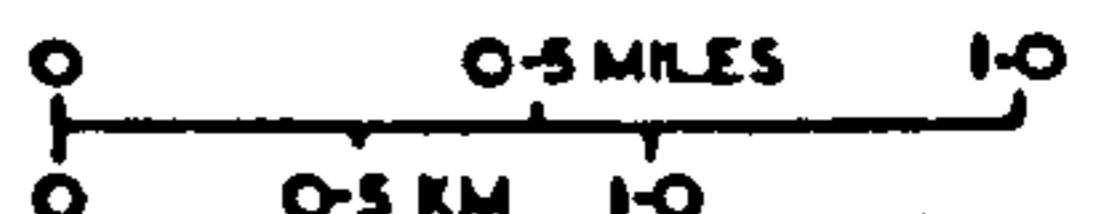
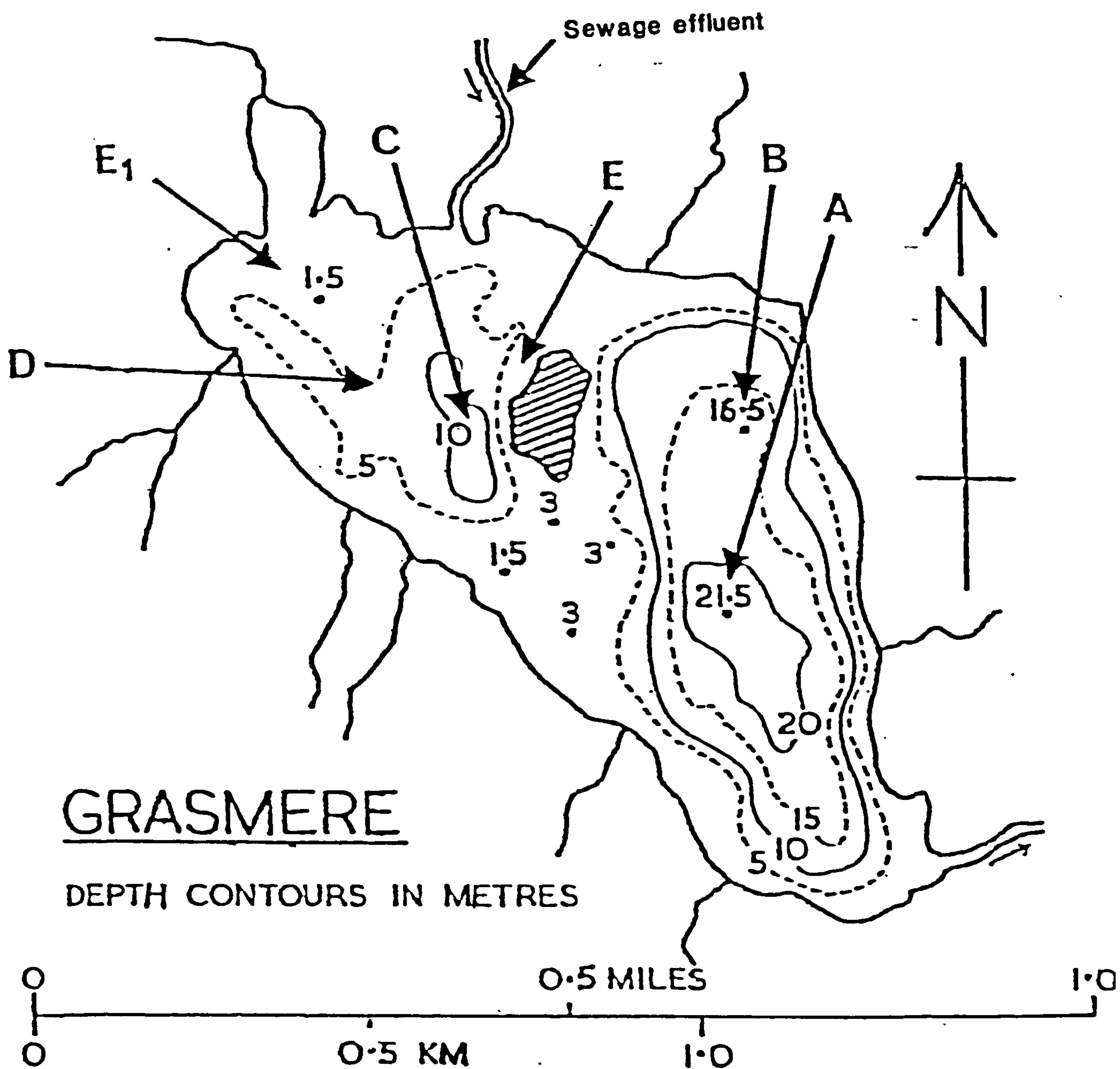


Fig. 4.3 Location of the sampling sites used in the present study on Grasmere.



Derwentwater and Brotherswater were similarly not marked and this presented some difficulty. The approximate position was ascertained by the use of prominent landmarks (three) and a distance rangefinder (Ranging Inc. Rochester, New York) with an echo sounder (Ferrograph : Graphic 240) being used to determine the appropriate depth. Using this system the position of the sampling point could be fixed to within  $\pm 20$  metres (horizontal distance) and therefore spatial variation of chemical parameters were investigated on these lakes.

#### 4.4 Sampling methods

Water samples were taken with a 5 l Friedinger water bottle. For the detailed surveys of Grasmere (1975 - 77) samples were taken at 1 metre intervals, the depth being measured from the water surface. During the inter-lake comparisons samples were taken 1 m and 5 m above the sediment water interface (measured from the sediment). Any microstratification in the samples taken for nitrification rate estimates (the sampler enclosed on 0.5 m column of water) was destroyed in one of two ways. In the detailed survey of Grasmere in 1977 a large sterile perspex block was placed into the sampler and the lid rapidly closed. The whole sampler was then inverted three or four times before dispensing sub-samples into sterile 135 ml glass bottles. Each bottle was flushed with approximately twice its own volume of water and sealed with suba-seals (Gallenkamp), taking care to exclude air bubbles. The perspex block was not resterilized between samples. During surveys for the inter-lake comparisons the whole of each 5 l sample was poured into another 5 l polythene container, shaken vigorously and sub-samples taken into 135 ml glass stoppered bottles.

Samples taken for chemical analysis during 1975 and 1976, or for experiments other than nitrification rate estimation, were dispensed directly into 5 l polythene containers and transported back to the laboratory. Sub-samples were taken into 1 or 0.5 l sterile glass bottles

(Sovirel) and sealed with screw caps or suba-seals.

When required pure methane (Chromatography Services Ltd) was added to completely filled 500 ml sample bottles sealed with suba-seals. Pressure was equalized by inserting a separate syringe needle. A calibration of volume of methane added to concentration of dissolved methane was prepared using temperature equilibrated filter sterilized water.

Sediment samples from the deep water sites of Grasmere (A, B, C and D) were taken with a Jenkin surface mud sampler with modified core tubes (see later). Samples from sites E and E<sub>1</sub> were obtained by hand using similarly designed core tubes. Duplicate samples were taken at each site and returned to the laboratory in the tubes.

Water temperature and dissolved oxygen concentrations were measured in the field with a combination thermistor/oxygen electrode (Mackereth 1964).

To reduce the analytical work load samples between 0 to 5 m, 5 to 10 m, 10 to 15 m and 15 to 20 m were bulked by adding equal volumes of each sample, between these depths, to a sterile bottle. These different strata were used because of the hydrographic data shown in Figure 4.1. The volume of each metre sample added was not adjusted to its relative volume within the lake.

Analysis of the samples from Grasmere began within three to four hours of sampling; for other lakes this time varied with distance from the laboratory. On no occasion did it exceed 6 hours.

#### 4.5 Physical measurements

##### 4.5.1 Water samples

The sample pH was measured by one of two methods depending upon the accuracy required. The less accurate method, which was used on the 'bulked' samples, where gaseous exchange and temperature changes had occurred, used a Radiometer GK 2401 C combination electrode and



Radiometer (pH 62) millivolt meter. For accurate pH measurements a 135 ml sample, which completely filled the bottle, was allowed to equilibrate, whilst stoppered, to room temperature. Using the above electrode and meter the probe was positioned approximately 3 cms from the bottom of the bottle whilst a teflon coated magnetic flea maintained a constant slow rate of stirring. Stable readings were obtained in approximately 3 minutes.

#### 4.5.2 Sediments samples

Physical measurements on, and the sampling of, discrete sediment layers was possible using modified Jenkin core tubes. The basal metal lid contained a central plate which could be pushed into the core tube simultaneously supporting the sediment core. After removing some of the overlying water the sediment core could be pushed up the tube with minimum disturbance of surface layers. This was further modified by using a piston and screw thread with a pitch of 2 mm, therefore the core could be moved up, or extruded from, the tube with reasonable accuracy (these modifications were developed by J.G. Jones of this laboratory).

Redox potential (Eh) measurements were taken with a bright platinum electrode (Radiometer Pl01) and saturated calomel reference electrode; the electrode couple being checked using ZoBells solution (1/300 m potassium ferricyanide plus 1/300 m potassium ferrocyanide in 0.1 m KCl, Hayes et al (1958)) in which a reading of approximately + 192 mV should be obtained. When required the platinum was polished using aluminium oxide powder (B.D.H. Chemicals) of about 0.05  $\mu\text{m}$  particle size. The reference electrode was first submerged in the overlying water whilst the platinum electrode was clamped firmly in the centre of the tube and the core extruded so that readings could be taken in the overlying water, at the sediment water interface and at 0.5 cm intervals into the mud.

The Eh reading was recorded one minute after the electrode had been positioned. The actual sediment redox potential was the measured potential plus that of the reference cell (+ 244 mV at 25°C).

pH measurements were obtained in a similar manner using a single glass microelectrode (Type No. SWRM/45/757, Russell pH Ltd) and a saturated calomel reference electrode positioned as for redox measurements. The use of this system eliminated any junction effects between the interface of the glass electrode and the sediments. Measurements were made in duplicate cores and the average values, at corresponding positions, calculated.

The core was extruded, at 0.5 cm intervals, and the sediment removed with the aid of a platform clamped to the top of the tube. The corresponding layers from the two cores were pooled and thoroughly mixed.

Dry weight analysis, was performed in duplicate, on two different but known volumes, after drying at 60°C for 48 hours.

Due to the particle and fibre content of sediments it is very difficult to accurately pipette a known volume. In this work a glass vial and a Finn pipette tip (Jencons) were weighed. The bore of the pipette tip was enlarged by removing the first few millimetres; the sediment was pipetted and the whole tip replaced in the vial for reweighing.

#### 4.6 Chemical analysis

##### 4.6.1 Water samples

###### Ammonia

Ammonia was determined by the indophenol blue method (after Chaney and Marbach, 1962) in which ammonia reacts with phenol and hypochlorite, in an alkaline solution, to form indophenol blue which is measured spectrophotometrically at 635 nm. The reaction is catalysed by nitroprusside. Ammonia free distilled water, for reagent preparation and blank determination, was obtained directly as double glass distilled water. All

reagents were stored at 4 - 5°C and glassware was cleaned in chromic acid.

#### Nitrate and nitrite

Nitrate, in an alkaline buffered solution, was reduced to nitrite using spongy cadmium (Elliot and Porter 1971). The nitrite was subsequently determined by diazotization with sulphanilamide and coupling the diazo compound with N-1-naphthylethylenediamine-dihydrochloride to form a red complex (Bendschneider and Robinson, 1952), which was measured spectrophotometrically at 543 nm.

The performance characteristics of these methods are shown in Figure 4.4.

#### Alkalinity

Alkalinity was measured by titration of a 50 ml water sample with 0.01 N hydrochloric acid (A.R. grade). The theory of this titration is briefly described by Mackereth et al (1978). The end point was located by means of a Gran plot (Talling 1973 in Mackereth et al, 1978) with the titre volume being recorded at pH values of approximately 4.6, 4.3 and 3.9. The titration was performed in a narrow necked 135 ml Pyrex bottle using a Metrohn 5 ml piston burette, the sample being constantly stirred with a teflon coated magnetic flea. The sample was allowed to equilibrate to room temperature in a completely filled bottle (no head space) but some CO<sub>2</sub> exchange could have occurred on sample transfer. These methods are described in detail by Mackereth et al (1978).

#### Total dissolved inorganic carbon

This value was required for the calculation of N-Serve sensitive carbon uptake (see later) and was obtained from the alkalinity and accurately measured pH value. Nomogram tables are available for direct estimation of this value (see for example Stumm and Morgan, p. 133, 1970)

but these take no account of the temperature dependence of pH and the dissociation constants involved in carbon dioxide and bicarbonate equilibria. Therefore the calculation was performed using temperature corrected values for pH, the first and second acidity constants ( $pK_1$  and  $pK_2$ ,  $H_2CO_3 = HCO_3^- + H^+$  and  $HCO_3^- = H^+ CO_3^{2-}$  respectively) and the ion product of water ( $pK_w$ ) (W. Davison pers. comm.). The correction was made to the temperature of incubation of the samples (see later). The values for the dissociation constants were obtained from Stumm and Morgan (1970) and the pH correction from a previously prepared calibration. The total carbon dioxide value (CT) thus obtained ( $m\ moles\ l^{-1}$ ) was converted to  $mg\ C\ l^{-1}$  from multiplication by the atomic weight of carbon.

Head space analysis was sometimes used to analyze for total dissolved  $CO_2$ . 25 ml of sample was drawn into a 50 ml polypropylene syringe, and the same volume of  $O_2$  free  $N_2$  was injected. The syringe needle was stoppered and 1 ml of 1.5 M  $H_2SO_4$  was injected through the butyl rubber head of the syringe, the whole shaken vigorously for 10 s, and then allowed to stand at room temperature for a further 2 minutes. The head space gases were subsampled through a 0.25 ml gas sampling valve on a Perkin-Elmer F30 gas chromatograph, passed through a short drying column of 14 - 22 mesh magnesium perchlorate at room temperature, and separated on a 2 m column of Carbosieve B at  $120^\circ C$ . The eluted gases were mixed with hydrogen and passed through a short nickel catalyst column at  $350^\circ C$ , where oxides of carbon were reduced to their hydrogenated equivalents. Thus  $CO_2$  was reduced to  $CH_4$  and detected with greater sensitivity by a flame ionization detector. Peak areas were integrated electronically. Analysis of standard bicarbonate solutions confirmed the linearity of the method and a 200  $\mu$  molar solution was used for performance assessment during sample analysis.

Fig. 4.4 Performance characteristics of the Indophenol blue method for ammonia determination, and cadmium reduction followed by diazotization for nitrate determination in natural lakewaters.

### Ammonia

Sensitivity  $1 \mu\text{g l}^{-1} \equiv 0.001163$  absorbance units

Limit of detection  $5 \mu\text{g l}^{-1}$ , (95% confidence level)

#### Precision measurements

Solution	Mean absorbance	Standard deviation $\mu\text{g l}^{-1}$		
		$s_w$	$s_b$	$s_t$
$0 \mu\text{g l}^{-1}$	0.005	0.9(6)	-	-
$56 \mu\text{g l}^{-1}$	0.068	2.1(6)	N.S.(5)	2.0(11)
$560 \mu\text{g l}^{-1}$	0.665	5.2(6)	10.9(5)	12.1(11)

N.S. = not statistically significant at the 5% probability level

\* Degrees of freedom are given in brackets.

### Nitrate

Sensitivity  $1 \text{ mg l}^{-1} \equiv 0.340$  absorbance units

Limit of Detection  $0.013 \text{ mg l}^{-1}$  (95% confidence level)

#### Precision measurements

Solution	Mean absorbance	Standard deviation $\mu\text{g l}^{-1}$			Concentration found/ $\mu\text{g l}^{-1}$	Recovery $\%$ <sup>**</sup>
		$s_w$	$s_b$	$s_t$		
$0 \mu\text{g l}^{-1}$	0.005	2.4(6)	-	-	-	-
$140 \mu\text{g l}^{-1}$	0.047	4.2(6)	7.2(5)	11.0(11)	-	-
$2100 \mu\text{g l}^{-1}$	0.726	27.9(6)	N.S.	41.6(11)	-	-
Esthwaite	0.156	8.8(5)	-	-	459	-
Esthwaite + $200 \mu\text{g l}^{-1}$	0.225	16.1(5)	-	-	662	$101.5 \pm 9.2$
Esthwaite + $2000 \mu\text{g l}^{-1}$	0.799	31.9(5)	-	-	2349	$94.5 \pm 1.7$

N.S. = not statistically significant at the 5% probability level

\* Degrees of freedom are given in brackets

\*\* 95% confidence limits also given

Comparison of the titration and chromatographic methods showed a variation of less than 6% at a concentration of  $23 \text{ mg C l}^{-1}$ .

#### Total dissolved methane

Total dissolved methane was estimated by head space analysis. A 25 ml water sample was drawn into a 50 ml polypropylene syringe and the same volume of helium injected. The syringe needle was stoppered and the whole vigorously shaken for 10 s and then allowed to stand at room temperature for a further 2 minutes. The head space gases were subsampled via a 5 ml gas sampling valve on a Hewlett-Packard 5710A Gas Chromatograph containing columns of chromosorb 102 or molecular sieve 5A. Methane was estimated in a thermal conductivity detector and peak area integrated electronically. The chromatograph was calibrated using standard  $\text{CH}_4$  gas mixtures (Phase Separations Ltd).

#### 4.6.2 Sediment samples

All analyses were carried out on the sediment interstitial water separated from the particulate fraction by centrifugation (M.S.E. Mistral 6L) at  $15,000 \times G$  for 10 minutes. The supernatant was immediately decanted and stored at  $4^\circ\text{C}$  until analysis.

Automated continuous flow procedures were used for all inorganic nitrogen determinands.

#### Nitrate and nitrite

Nitrate was reduced to nitrite with hydrazine sulphate, under alkaline conditions, in the presence of  $\text{Cu}^{2+}$  as a catalyst, and  $\text{Zn}^{2+}$  to counteract interferences from natural waters (Downes, 1978). The nitrite was subsequently detected as described for water samples. The reduction stage was omitted during nitrite determination.

### Ammonia

This method was adapted from that of Crooke and Simpson (1971) and is described in detail by Jones et al (1980).

The recovery and performance characteristics of the methods for the ammonia and nitrate analyses are presented by Jones et al (1980).

### Alkalinity

Alkalinity was determined as described for water samples but using smaller sample volumes. Total CO<sub>2</sub> was determined by calculation using the temperature corrected pH and dissociation constant values. The temperature used for correction was the incubation temperature used for the samples and not 'in situ' values.

## 4.7 Nitrification rate estimation

All measurements were made using the <sup>14</sup>C-bicarbonate method described by Billen (1976) in which the CO<sub>2</sub> uptake between samples treated with an lithotrophic nitrification inhibitor, and untreated samples is compared. The difference being attributed to nitrifying activity. The inhibitor used was N-Serve (2-chloro, 6-trichloromethyl pyridine) dissolved in absolute alcohol (ethanol) and sterilized by filtration through 0.22 μ polycarbonate (Nucleopore) membranes. All inhibitor solutions were freshly prepared in glass scintillation vials with Bakelite tops and metal foil lined inserts.

### 4.7.1 Water samples

Water samples were taken and any microstratification disrupted as previously described. In the detailed survey of Grasmere six 135 ml bottles were filled, flushing with approximately twice their own volume, and stoppered with No. 37 suba-seals taking care to exclude air bubbles. Two bottles were treated immediately with the N-Serve solution and another two received an equivalent volume of ethanol. The remainder were treated

with neutralised formalin to a final concentration of 2% (which has been shown to completely inhibit biological activity in such samples, J.G. Jones pers. comm.). The samples were transported back to the laboratory in the dark and equilibrated in a water bath set at 10°C. After equilibration 5 micro Curies (185 k Bq) of <sup>14</sup>C-bicarbonate (approximately 59 m Curies per m mole specific activity, Radiochemical Centre, Amersham) were added to each bottle and incubation continued for a further 19 to 20 hours.

After incubation duplicate 50 ml aliquots, from each bottle, were filtered, through 0.22 µm, 25 mm Millipore membranes, on a 12-channel Millipore filter manifold. Whilst still under vacuum the filters were rinsed with approximately 2 ml of 2% (V/V) hydrochloric acid to remove uncombined bicarbonate and placed in a scintillation fluor (see below) which dissolved the cellulose ester membranes. The samples were then counted on a Packard 3302 or Tracor 6882 model scintillation counter. In the former, quench correction was by sample channels ratio whilst in the latter the d.p.m. was calculated automatically using relative pulse heights.

The scintillation fluor used to dissolve cellulose ester membranes was made up as follows:

1.4 - Dioxan	900 ml	(Scintillation grade)
Napthalene	100 g	(Scintillation grade)
Butyl-PBD	7 g	" "
bis - MSB	0.5 g	" "
Water	100 ml	(Single distilled)

For the inter-lakes comparison the procedure was essentially the same but with the following modifications.

The method detects a difference between two separate treatments; correction of the values for non-biological uptake (formalin treatment) does not affect this difference and so the control samples were eliminated enabling more replicate treatments to be used.



The water samples were mixed as previously described and dispensed into twelve 135 ml bottles. Six of these were treated with N-Serve and the remaining six receiving an equivalent volume of ethanol, before stoppering with ground glass tops. The all glass bottle systems prevented any adsorption of N-Serve to the butyl rubber septa (see Bremner et al (1978)). On arrival at the laboratory, incubation, at 10°C, was started until temperature equilibration. 5 micro Curies (185 k Bq) of <sup>14</sup>C-bicarbonate were then added to each bottle and incubation continued for three to four hours. The samples were then filtered and counted as previously described.

#### Calculation of CO<sub>2</sub> uptake

The theory of the use of <sup>14</sup>C-bicarbonate, to measure carbon dioxide uptake, is well established and so will not be described here; the reader is referred to I.B.P. Handbook No. 12 for full details. The calculation can be described using the general formula.

$$X = \frac{A}{B} \times C \times F_1 \times F_2 \times F_3$$

where X = Carbon uptake per unit volume per unit time

A = <sup>14</sup>C taken up by a unit volume of sample

B = <sup>14</sup>C added to a unit volume of sample

C = Total inorganic carbon available per unit volume of sample

F<sub>1</sub> = Isotope correction factor

F<sub>2</sub> = Time correction factor

F<sub>3</sub> = Volume correction

It should be noted that the radiotracer additions are in terms of carbon and therefore the total inorganic carbon available should be in the same units.

The <sup>14</sup>C solution was calibrated for each sampling date by addition of a known volume of scintillation fluor 2 (see below) containing

phenethylamine to absorb the  $\text{CO}_2$  (see Herbland, 1977).

#### 4.7.2 Sediment samples

Due to the difficulties of accuracy and reproducibility in pipetting sediment slurries all sub-samples were treated individually on a weight basis. Aliquots ( $\sim 2$  ml) from the separate 0.5 cm depth layers of sediment were placed in six pre-weighed, clear glass, 15 ml hypovials (Pierce Chemical Co., Rockford, Illinois) and reweighed. The vials were then sealed with either butyl rubber or neoprene septa, held in position by crimped aluminium seals. N-Serve (in ethanol) was added to half the vials; the remainder received an equivalent volume of ethanol and allowed to equilibrate to the incubation temperatures used. These were  $10^\circ\text{C}$  for sites A, B and C and  $15^\circ\text{C}$  for sites D, E and  $E_1$ . The littoral sediments from sites D, E and  $E_1$  were also incubated in the dark. After temperature equilibration (approximately 30 minutes)  $5 \mu$  Curies (185 k Bq) of  $^{14}\text{C}$ -bicarbonate (specific activity  $59 \text{ m Ci m mole}^{-1}$ ) were added to each sample and incubation continued for 3 to 4 hours. The aluminium seals were removed and an amount of sediment ( $\sim 50\%$  of sample), from each hypovial, placed into a Pankhurst tube, the side arm of which contained approximately  $500 \mu\text{l}$  of phenethylamine (Koch Light : Scintillation grade). The tubes were then sealed with suba-seals. A few micro-litres of  $10\%$  hydrochloric acid were added to the sediment to drive off any uncombined  $^{14}\text{C}$ -bicarbonate. A small volume of acid was used to avoid alteration of the ratio of liquid to particulate matter. The released  $^{14}\text{CO}_2$  (plus any unlabelled  $\text{CO}_2$ ) was trapped in the phenethylamine and this ensured that the equilibrium, between the air and liquid partition of  $\text{CO}_2$ , was in favour of the gaseous form. This removal was relatively rapid but the reaction was allowed to proceed for 60 minutes (99.7% recovery after 30 minutes).

Aliquots of the  $\text{CO}_2$  free sediment slurry were then removed into combustible polycarbonate capsules (Intertechnique Ltd), capped and

weighed. The weight of the capsule and cap was negligible and therefore this weight corresponded to the amount of sediment to be analysed for  $^{14}\text{C}$  content. Duplicate aliquots were taken from each sample. Carbon-14 was extracted from the samples by oxidation in an Intertechnique Model IN4101 sample oxidiser, the resulting  $^{14}\text{CO}_2$  being trapped in a scintillation fluor containing phenethylamine. The product of  $\text{CO}_2$  adsorption is phenethylamine carbamate which is insoluble in the primary solvent (toluene) therefore methanol (A.R. grade) was added as a secondary solvent.

The scintillation fluor used for  $\text{CO}_2$  adsorption therefore contained

Toluene	500 ml	(Scintillation grade)
Phenethylamine	250 ml	" "
Methanol	250 ml	(A.R. grade)
P.P.O.	5 g	
P.O.P.O.P.	0.5 g	

The efficiency of oxidation was estimated using unlabelled sediment and a known amount of either  $^{14}\text{C}$ -hexadecane standard or  $^{14}\text{C}$  sample oxidiser standards (Radiochemical Centre, Amersham). The samples were counted as previously described.

All the samples would be subject to the same oxidation efficiency and counting efficiency (constant quench) so the measured efficiency from the standards (6 replicates) was used to correct all the counts to d.p.m. values. The measured efficiencies were  $64 \pm 2\%$ .

#### Calculation of $\text{CO}_2$ uptake rates

The  $\text{CO}_2$  uptake rate for each bottle had to be calculated separately. From the known weight of sediment oxidised and its radioactivity, the mean d.p.m. per g wet weight was calculated for the duplicate aliquots oxidised. From this value and the known wet weight of sediment in the initial exposure bottle this mean d.p.m. was converted to a whole sample value. From the dry weight analysis the percentage of interstitial-

water in a known weight of wet sediment was calculated. From the CT value (converted to mg carbon per litre, see page 98 ) the total inorganic carbon available in the initial exposure bottle was also calculated. The total carbon uptake rate was determined using the equation described on page 98. This value is converted to a gram dry weight basis using the figures from the dry weight analysis again. The inorganic carbon uptake thus expressed as mg. C g<sup>-1</sup> dry wt allows direct comparison of results.

Thus if:

C = wet weight of whole sample (g.)

B = weight of sample oxidised (g.)

A = d.p.m. of sample oxidised (d.p.m.)

M = % of sample which is interstitial water (assume 1 ml = 1 g) (ml)

E = inorganic C in interstitial water (mg C l<sup>-1</sup>)

Z = <sup>14</sup>C added to sample (d.p.m.)

Then i)  $\frac{1.0}{B} \times A = \text{dpm per g wet weight} = X$

ii)  $C \times X = \text{whole sample d.p.m.} = Y$

iii)  $C \times \frac{M}{100} = \text{interstitial water content of sample} = D \text{ (ml)}$

iv)  $\frac{E}{1000} \times D = \text{inorganic carbon in sample} = W$

v)  $\frac{Y}{Z} \times W \times F_1 \times F_2 = \text{C uptake rate per whole sample per unit time} = P.$

$F_1 = \text{isotope correction factor}$

$F_2 = \text{time correction factor.}$

Then P is corrected to g dry weight from the dry weight analysis.

#### 4.8 Analysis of results

Students 't' test was used to identify significant differences between the mean values of the N-Serve and alcohol treatments. For the water samples the two aliquots from each bottle were used as separate

data sets. For sediments the mean values from each bottle were used.

For both water and sediments the N-Serve sensitive carbon uptake was converted to values for nitrogen oxidised by multiplication by the factor 9.01. This is the mean value of the factors 8.3 and 9.72 used by Billen (1976) and Christofi (1978).

#### 4.9 Bacteriological methods

##### 4.9.1 Culture medium

The basal mineral salts medium of Skerman (described in Collins (1969)) was used throughout this study. The preparation was as described by Collins (1969) and is illustrated in Table 4.1 with a few modifications. The substrates, ammonium sulphate or sodium nitrite, were not added until after sterilization and subsequent addition was only in concentrations of 1 to 400 mg N l<sup>-1</sup>.

The pH was adjusted to 7.6 after sterilization (121°C 15 minutes) and substrate addition. Where dilutions of the basal medium were required double glass distilled water was used, with sterilization, substrate addition and pH adjustment being carried out after dilution.

##### 4.9.2 Isolation of nitrifiers

The media were distributed in 150 to 200 ml volumes into 250 ml Erlenmeyer flasks and inoculated with 10 ml of lakewater. Incubation was at 20°C in an orbital incubator (Gallenkamp) set at 130 to 150 r.p.m. All treatments were carried out in triplicate and frequent spot tests were made for ammonia, nitrite and nitrate.

##### 4.9.3 Counting of nitrifiers

The medium used was the most efficient formulation found in isolation experiments (see results). Three experimental designs of the Most Probable Number technique were used

i) Tenfold dilution interval with 5 replicates per dilution. The

Table 4.1. Basal medium composition, and preparation procedure, of the medium used for the culture of lithotrophic nitrifying organisms (from Collins, 1969).

**TABLE I**  
Solutions required for the preparation of various mineral salts media, Group A (from Skerman, 1967)

Solution	Amount	Procedure
1. N NaOH	1 litre	Sterilize at 121°C for 20 min
2. 0.074 M H <sub>3</sub> PO <sub>4</sub>	1 litre	Sterilize at 121°C for 20 min
3. Solution from 2, above	200 ml	Dilute to 2000 ml (0.0074 M); sterilize as for Solution 1
4. Solution from 3, above	1 litre	Neutralize with use of N NaOH; sterilize as for Solution 1
5. NaHCO <sub>3</sub> in 100 ml water	8.333 g	Sterilize as for Solution 1
6. CaCl <sub>2</sub> in 100 ml water	5.0 g	Sterilize as for Solution 1
7. NaNO <sub>3</sub> in 100 ml water	5.0 g	Sterilize as for Solution 1
8. Glucose in 100 ml water	10.0 g	Sterilize at 110°C for 25 min
9. Mannitol in 100 ml water	10.0 g	Sterilize at 110°C for 25 min
10. Sucrose in 100 ml water	10.0 g	Sterilize at 110°C for 25 min
11. Sodium citrate in 100 ml water	2.0 g	Sterilize at 121°C for 20 min
	anhydrous (or 2.77 g hydrated)	
12. Phenol in 100 ml water	10 g	Sterilize at 121°C for 20 min
13. Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> in 100 ml water	10 g	Sterilize at 121°C for 20 min
14. 0.5 M HCl	100 ml	Sterilize at 121°C for 20 min
15. 0.0167 M H <sub>3</sub> PO <sub>4</sub>	1 litre	Neutralize 500 ml with N NaOH; sterilize as for Solution 11
16. Monoethylamine hydrochloride in 100 ml water	5 ml	Sterilize by filtration

**TABLE II**  
Solutions required for the preparation of various mineral salts media, Group B (from Skerman, 1967)

Solution	Amount per 100 ml solvent	Solvent required	Final concentration (µg/litre medium)
1. NaCl	3.0 g	0.0074 M H <sub>3</sub> PO <sub>4</sub>	300,000
2. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	6.6 g	0.074 M H <sub>3</sub> PO <sub>4</sub>	660,000
3. LiCl <sub>2</sub>	21.0 mg	0.0074 M H <sub>3</sub> PO <sub>4</sub>	21
4. CuSO <sub>4</sub> .5H <sub>2</sub> O	80.0 mg	0.0074 M H <sub>3</sub> PO <sub>4</sub>	80
5. ZnSO <sub>4</sub> .7H <sub>2</sub> O	106.0 mg	0.0074 M H <sub>3</sub> PO <sub>4</sub>	106
6. H <sub>2</sub> BO <sub>3</sub>	600.0 mg	0.0074 M H <sub>3</sub> PO <sub>4</sub>	600
7. Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> .18H <sub>2</sub> O	123.0 mg	0.0074 M H <sub>3</sub> PO <sub>4</sub>	123
8. NiCl <sub>2</sub> .6H <sub>2</sub> O	110.0 mg	0.0074 M H <sub>3</sub> PO <sub>4</sub>	110
9. CoSO <sub>4</sub> .7H <sub>2</sub> O	109.0 mg	0.0074 M H <sub>3</sub> PO <sub>4</sub>	109
10. TiCl <sub>4</sub>	60.0 mg	0.074 M H <sub>3</sub> PO <sub>4</sub>	60
11. KBr	30.0 mg	Water	30
12. KI	30.0 mg	Water	30
13. MnCl <sub>2</sub> .4H <sub>2</sub> O	629.0 mg	0.074 M H <sub>3</sub> PO <sub>4</sub>	629
14. MgSO <sub>4</sub> .7H <sub>2</sub> O	1.4 g	Water	140,000
15. SnCl <sub>2</sub> .2H <sub>2</sub> O	36.0 mg	Water	36
16. FeSO <sub>4</sub> .7H <sub>2</sub> O	300.0 mg	Water	300

Preparation of the basal mineral salts medium is carried out as follows—

*Step 1.* Pipette into a 1 litre standard flask the following amounts of solutions from Group B: 10.0 ml of Solutions 1 and 2 and 0.1 ml of each of Solutions 3–10.

*Step 2.* Add approximately 600 ml of 0.0074 M H<sub>3</sub>PO<sub>4</sub> (Solution 3, Group A) and 210 ml of water.

*Step 3.* Adjust the pH to 7.0 with N NaOH (Solution 1, Group A).

*Step 4.* Add 0.1 ml of Solutions 11 and 12 from Group B.

*Step 5.* Take 0.1 ml of the MnCl<sub>2</sub> solution (Solution 13, Group B), add 9.9 ml of 0.074 M H<sub>3</sub>PO<sub>4</sub> (Solution 2, Group A), and adjust the pH to 7.0. Autoclave and filter. Add the filtrate to the medium.

*Step 6.* Add 10 ml of Solution 14, Group B, and 0.1 ml of 15 and 16, Group B.

*Step 7.* Using the neutralized 0.0074 M H<sub>3</sub>PO<sub>4</sub> (Solution 4, Group A), make the final volume to 1 litre.

*Step 8.* Sterilize at 121°C for 20 min.

medium was dispensed in 20 ml volumes into 16 x 150 mm rimless Pyrex test tubes. The inoculation volume was 1 ml and incubation prolonged for 100 days, with periodic spot tests for nitrite and nitrate. Ammonium sulphate was readded 14 days before final chemical analysis. This design was only used for water samples.

ii) A 1:2 dilution interval with eight replicates per dilution, carried out in 8 x 12 microtiter dishes (Sterilin). The dilution was obtained using 150  $\mu$ l aliquots of medium and 150  $\mu$ l inocula dispensed with an eight channel Titertek (Jencons) piston pipette.

iii) A 1:4 dilution interval with eight replicates per dilution again carried out in 8 x 12 microtiter dishes. The dilution was obtained using 150  $\mu$ l aliquots of medium and 50  $\mu$ l inocula again using 8 channel Titertek piston pipettes.

The microtiter dishes were covered with plastic lids and placed in polythene bags to which 1 ml of water had been added to maintain humidity and reduce evaporation. Incubation was at 20°C for 100 days before analysis for nitrite and nitrate.

#### 4.9.4 Spot tests

##### Ammonia

The indophenol method described on page 91 was used with reagent volumes scaled down to match sample volumes.

##### Nitrite and nitrate

Nitrite was detected by the diazotization reaction described on page 92. If this was negative spongy cadmium was added to test for nitrate.

#### 4.9.5 Enrichment cultures

Enrichment cultures of nitrifiers were prepared from either lakewater, or sediments diluted with filter sterilized lake water, by addition of 0.2 to 0.5 g l<sup>-1</sup> ammonium sulphate (A.R. grade) and aerating with compressed air. Nitrate accumulation was noted within two to thirty days of incubation at room temperature.

Enrichment cultures of heterotrophic bacteria were obtained by adding approximately 0.1 g Bacto Peptone (Difco or Oxoid) and 0.1 g D-Glucose (A.R. grade) to lakewater and incubating overnight, without shaking, at room temperature.

#### 4.9.6 Direct counts of bacteria

Counts were performed by epifluorescence microscopy, on black membrane filters, using the procedure described by Jones and Simon (1975). The stain was Acridine orange at a final concentration of 10 mg l<sup>-1</sup> sample and a contact time of 5 minutes. Five to 15 bacteria per field were counted and the total count exceeded 400 bacteria. This procedure gave 95% confidence limits to the actual count of  $\pm 10\%$ .



## CHAPTER 5

## Results and Discussion

This chapter will be divided into sections each dealing with a different aspect of the present work. Each section will conclude with a discussion of its contents and a general discussion will follow in the final chapter.

## 5.1 Seasonal cycles of physical and chemical parameters in Grasmere

On each sampling date temperature, dissolved oxygen readings and water samples were taken at 1 metre depth intervals at the 20 m site (A) on Grasmere. Ammonia, nitrite and nitrate determinations were carried out on integral samples, prepared as described on page 89. For comparison, temperature and oxygen concentrations were calculated as the mean of the values between 0 - 5, 5 - 10, 10 - 15 and 15 - 20 m respectively. The 5, 10 and 15 m depths were thus duplicated. The seasonal cycles of temperature, dissolved oxygen, ammonia and nitrate concentrations for 0 - 5, 5 - 10, 10 - 15 and 15 - 20 m depth layers are shown in Figures 5.1A, B, C and D respectively. Nitrogen gas determinations were not performed, nitrite was never detected in significant concentrations and therefore the inorganic determinands were restricted to ammonia and nitrate. The pH values for the bulked samples were also determined but due to gaseous exchange and temperature changes during the sampling mixing procedure, these bear little relation to in situ values and so are not reported. The 0 - 5, 5 - 10, 10 - 15 and 15 - 20 m depth layers can be defined, for convenience, as the epilimnion, metalimnion, upper and lower hypolimnion respectively.

## Seasonal temperature cycle

As would be expected the epilimnion and metalimnion show seasonal trends which depend upon the prevailing climate. The highest temperatures

Figure 5.1 Seasonal variations, presented as monthly mean values, of temperature, dissolved oxygen concentrations and ammonia and nitrate concentrations between different depth layers in Grasmere during 1976 and 1977.

Figure 5.1A 0 to 5 m 1) Temperature ( $^{\circ}\text{C}$ )  
 2) Dissolved oxygen concentration ( $\text{mg l}^{-1}$ )  
 3) Ammonia and nitrate concentrations ( $\mu\text{g N l}^{-1}$ )

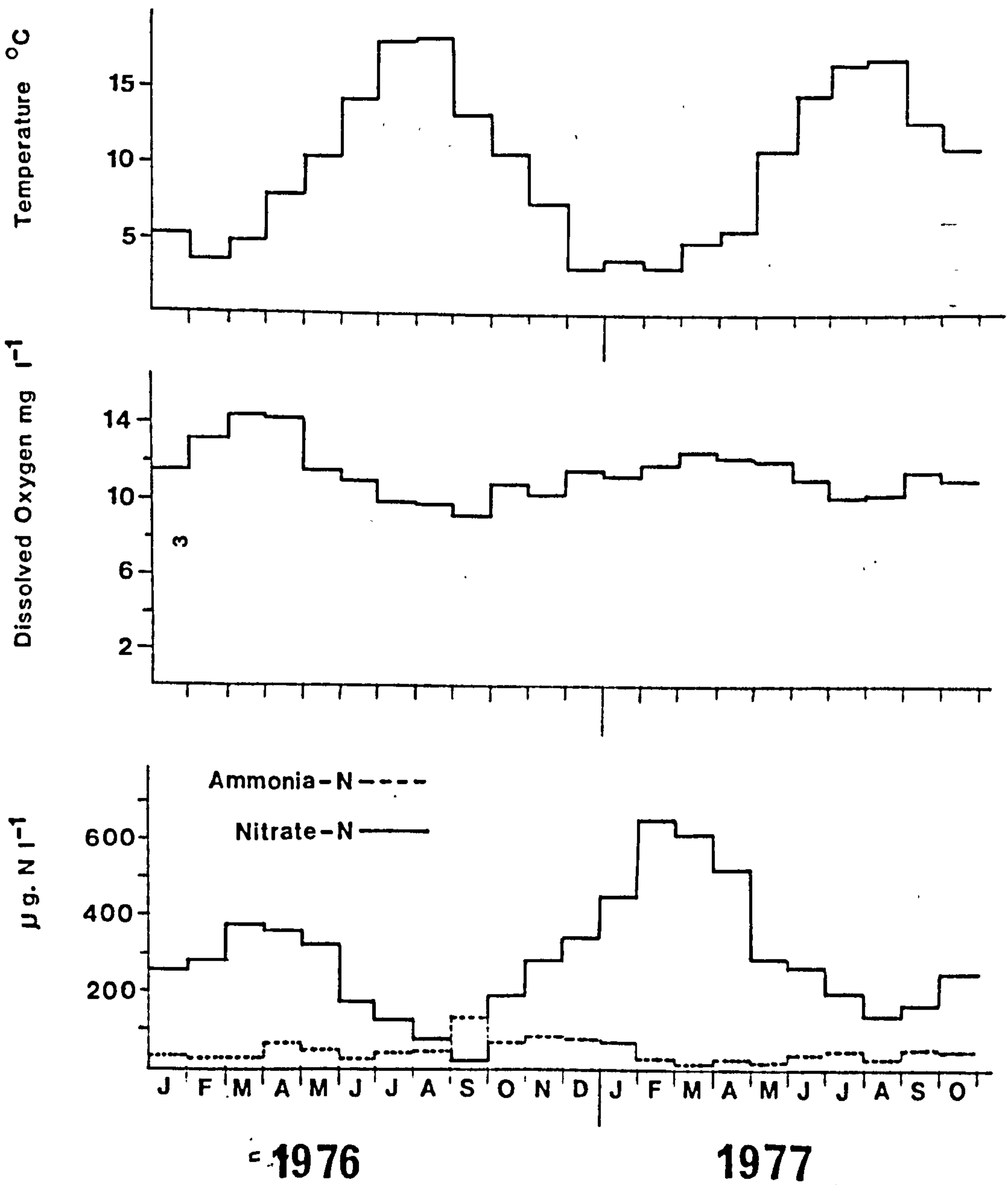


Figure 5.1B 5 to 10 m 1) Temperature ( $^{\circ}\text{C}$ )  
 2) Dissolved oxygen concentration ( $\text{mg l}^{-1}$ )  
 3) Ammonia and nitrate concentrations ( $\mu\text{g N l}^{-1}$ )

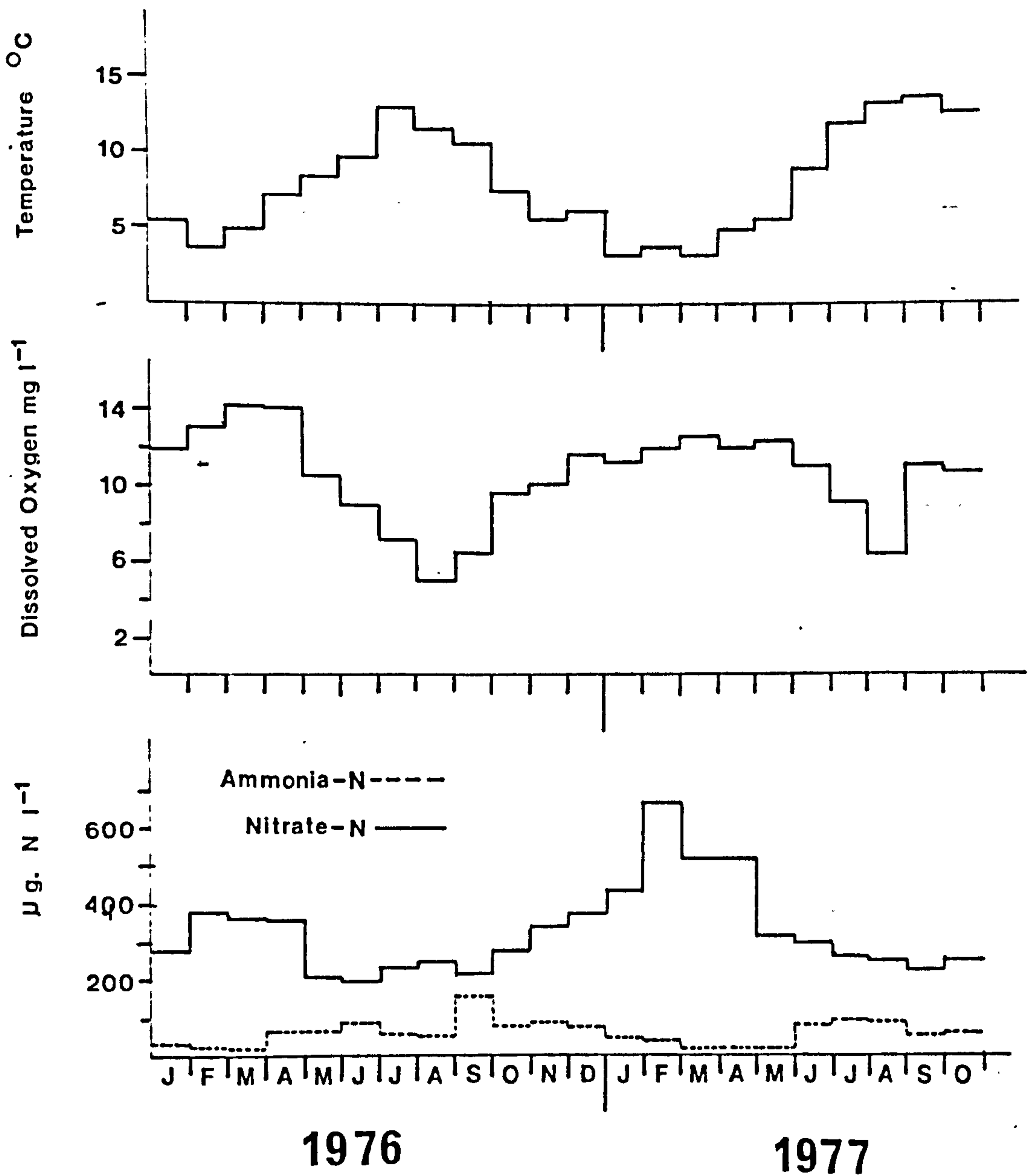


Figure 5.1C 10 to 15 m 1) Temperature ( $^{\circ}\text{C}$ )  
 2) Dissolved oxygen concentrations ( $\text{mg l}^{-1}$ )  
 3) Ammonia and nitrate concentrations ( $\mu\text{g N l}^{-1}$ )

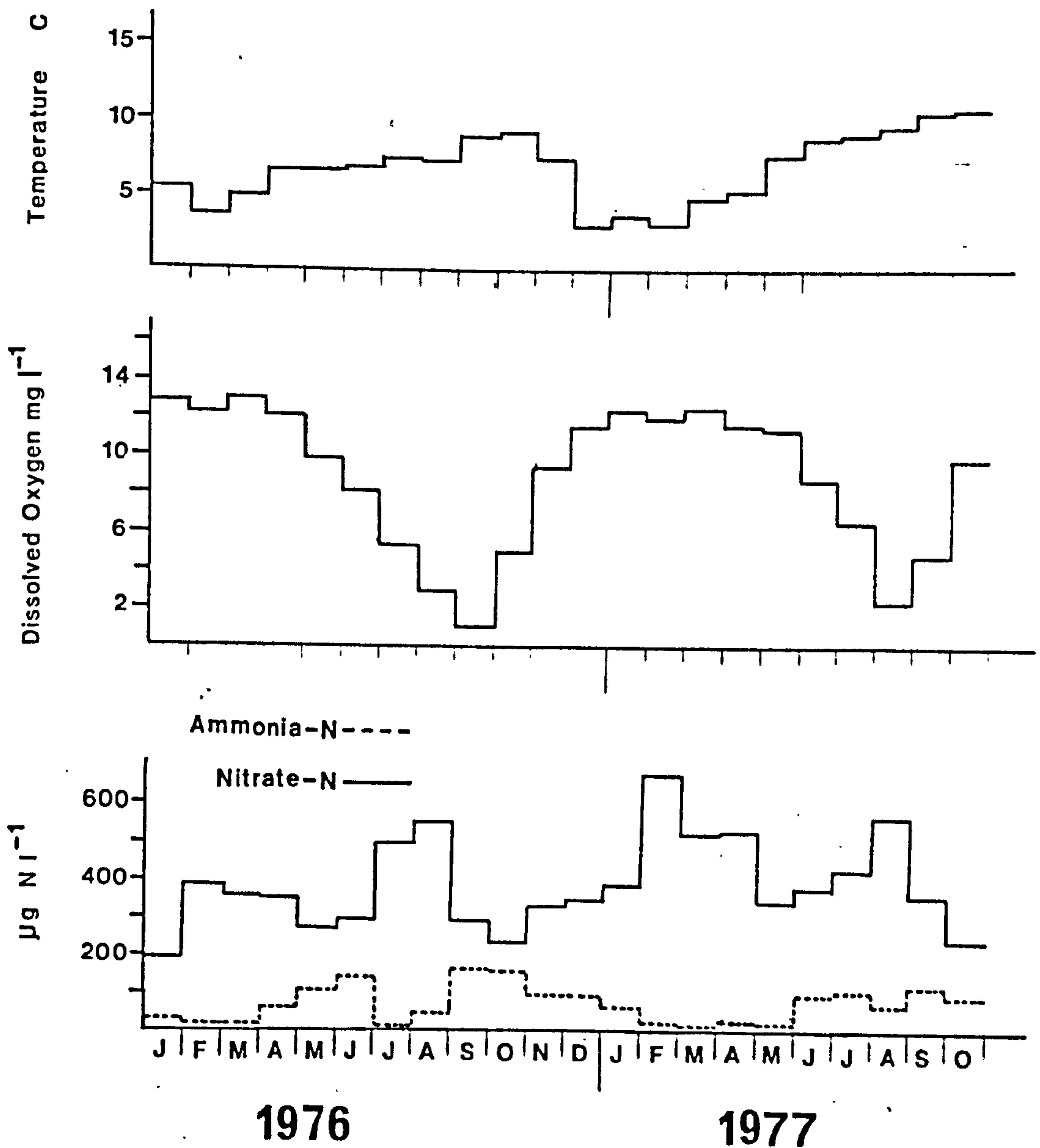
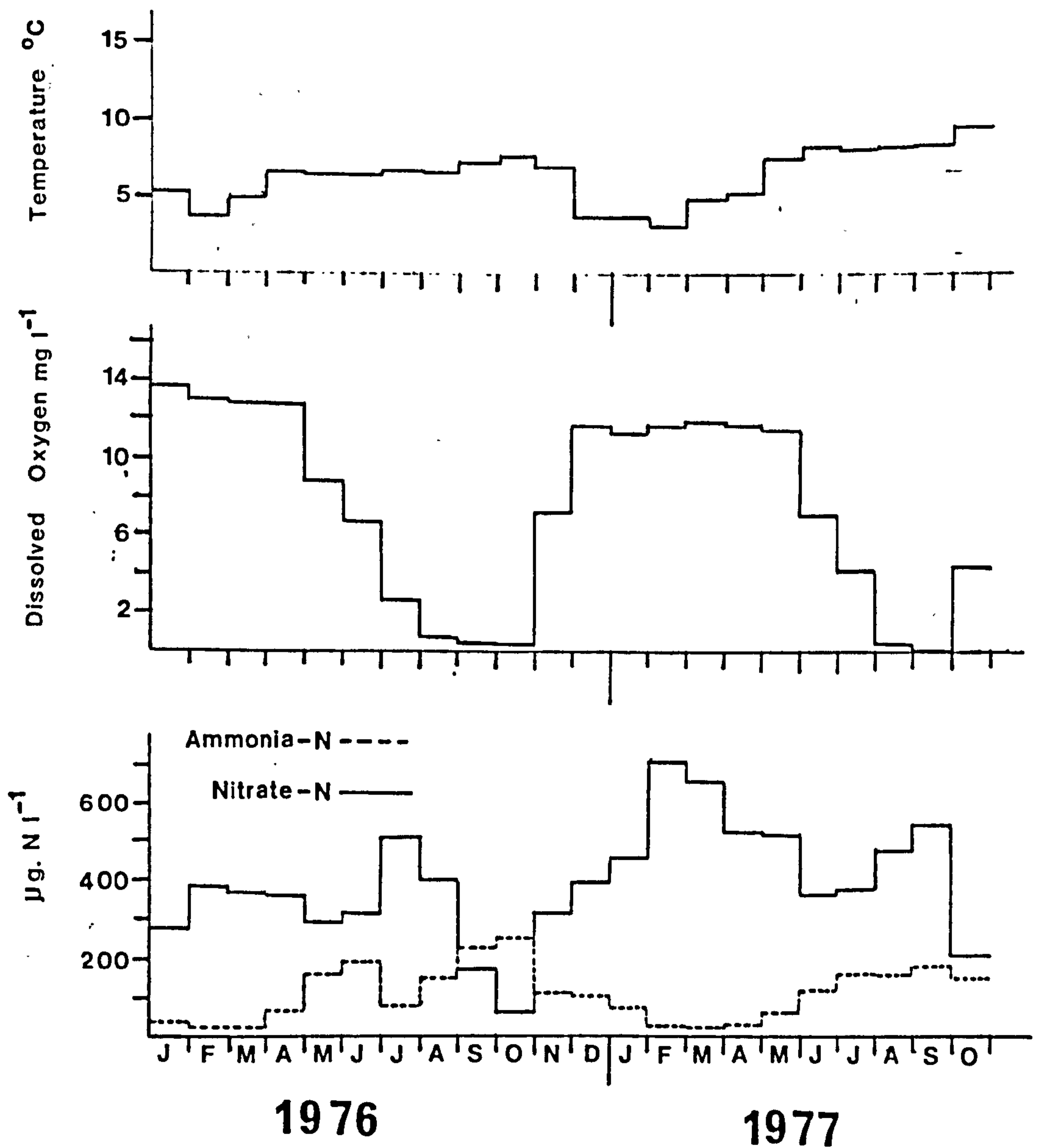


Figure 5.1D 15 to 20 m 1) Temperature ( $^{\circ}\text{C}$ )  
 2) Dissolved oxygen concentrations ( $\text{mg l}^{-1}$ )  
 3) Ammonia and nitrate concentrations ( $\mu\text{g N l}^{-1}$ )



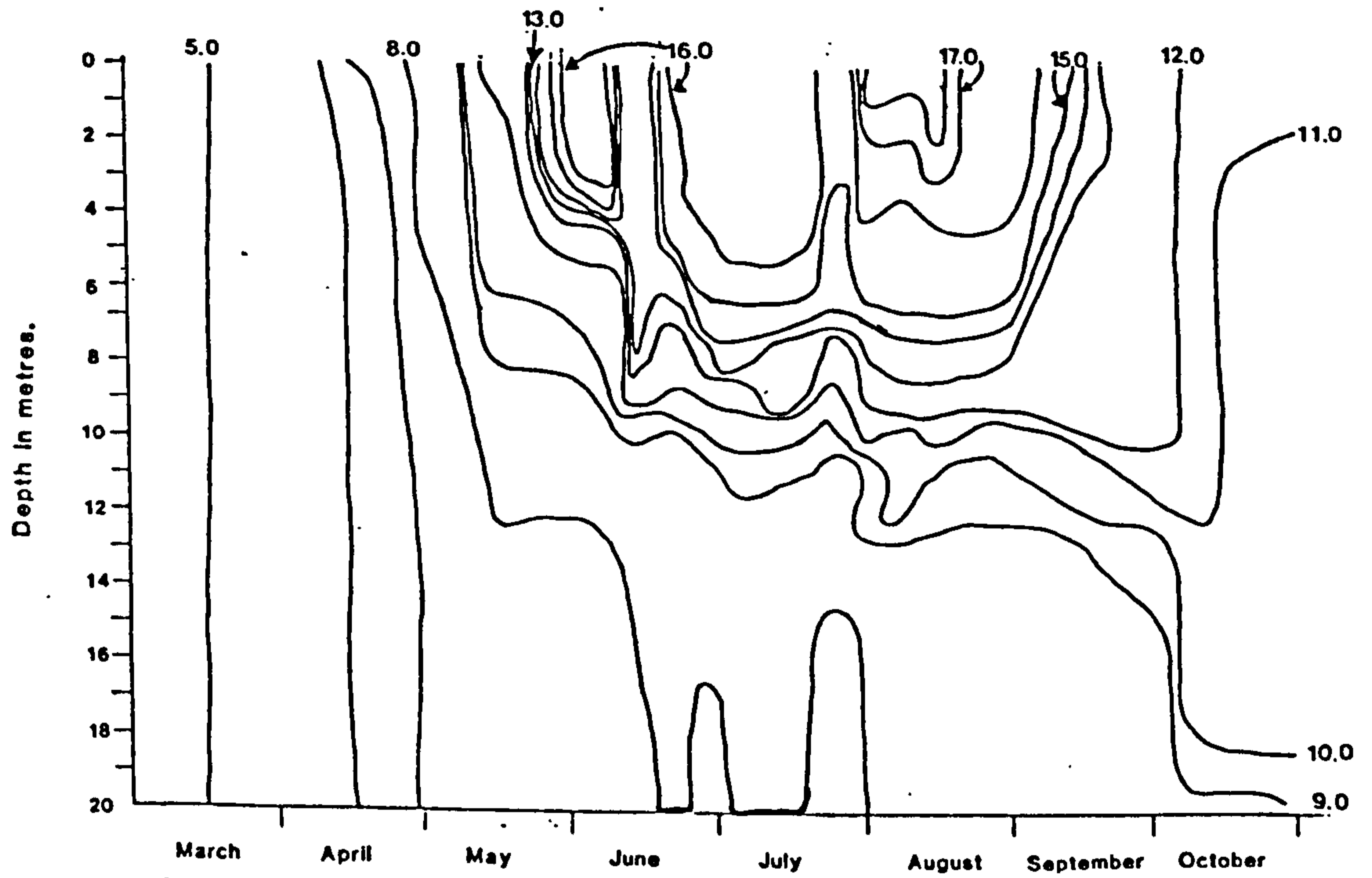
were recorded in surface waters. During 1976 and 1977 the monthly mean temperature of the epilimnion varied between 3°C and 18°C. Thermal stratification usually became established in April and autumnal overturn occurred during September or October. The temperature variation in the upper and lower hypolimnion, and therefore profundal sediments, was small with less than 8°C difference over the two years reported. The isotherm diagram for 1977 is shown in Figure 5.2. It can be seen that the boundaries for the thermal zones correspond well with the defined depth intervals given above.

#### Seasonal dissolved oxygen cycles

The seasonal cycles for dissolved oxygen were typical of those found in stratified eutrophic lakes in the temperate zone. Epilimnetic concentrations showed little change over the seasons and supersaturation, due to photosynthetic activity, was often recorded. In the hypolimnion deoxygenation was apparent during summer stratification with the 15 to 20 m layer becoming totally anoxic. The upper hypolimnion also showed a marked oxygen deficit but never reached total anoxia. However, the mixing of discrete samples, to form an integral over depth, does result in some loss of information. For example, during the years 1976 and 1977, complete deoxygenation was recorded at 13 m depth and below with a clearly defined oxycline between 12 and 13 m.

Hypolimnetic areal deficits (as  $\text{mg O}_2 \text{ m}^{-2} \text{ day}^{-1}$ ) based on i) average oxygen consumption rates over the period of stratification and ii) initial consumption rates over the first four weeks of stratification, with and without correction for turbulent inputs from the metalimnion, for the years 1969 to 1976, are presented by Hall *et al* (1978). The mean value for this period was  $433 \text{ mg O}_2 \text{ m}^{-2} \text{ day}^{-1}$ , with a range of 122 to  $1090 \text{ mg O}_2 \text{ m}^{-2} \text{ day}^{-1}$ . There was a marked increase in deoxygenation of the hypolimnion following discharge of the sewage effluent into the lake in 1971.

Figure 5.2 Isotherm diagram (based on weekly temperature measurement at 1 m depth intervals) in Grasmere 1977 (Contours in  $^{\circ}\text{C}$ )





The percentage of hypolimnion which became deoxygenated prior to 1971 was less than 10%. Between 1971 and 1976 this percentage increased to 77%. Although there were decreases in the areal deficits between 1974 and 1976 it is not possible to speculate if this was a recovery process, an improvement in effluent quality or merely a reflection of differences in climate.

#### Seasonal cycles of inorganic nitrogen

The seasonal cycles of ammonia and nitrate in the epilimnion and lower hypolimnion (Fig. 5.1A and D) in 1976 and 1977 show much the same pattern as for 1975 described by Hall et al (1978). In almost all samples nitrate was the dominant species whilst in the main inflow (River Rothay) ammonia was predominant due to enrichment by the sewage effluent (see Hall et al 1978). Inflow data are not presented here. Comprehensive field nitrogen data were obtained from 1975 to 1977 and over this period differences in gross seasonal fluctuations in concentrations were observed. However, on the basis of this limited annual data it is not possible to speculate if these differences are real or part of the inherent variability within the lake. Actual concentration changes will therefore only be discussed when directly relevant. This discussion will deal with consistent seasonal cycles observed over the three years.

#### Ammonia

Prior to stratification ammonia is present at equal concentrations throughout the depth profile ( $10 - 20 \mu\text{g N l}^{-1}$ ). This develops into an inverse clinograde distribution (increasing concentrations with depth) with the onset of stratification due to assimilation in the epilimnion, and increasing concentrations in the hypolimnion. As the hypolimnion was aerobic this increase is presumably due to nitrogen mineralization processes. In June, or July, the hypolimnetic concentration rapidly declines producing a clinograde distribution (concentrations decreasing

with depth) which prevails until the water becomes anoxic and ammonia accumulates to maximal concentrations ( $200 - 250 \mu\text{g N l}^{-1}$ ) reestablishing the inverse clinograde distribution. Upon overturn ammonia is redistributed throughout the lake ( $100 - 150 \mu\text{g N l}^{-1}$ ) and the concentration then declines to reach minimal values in January or February. The accumulation of ammonia in anoxic hypolimnetic waters has been recognised for a number of years and is thought to be due to release from reduced sediments (Hutchinson, 1957). Although high concentrations of ammonia were found in oxidised sediment interstitial water (see later), which presumably is freely mobile, it is clear that an oxidised sediment-water interface presents a barrier to ammonia exchange with overlying water. It is not clear whether this barrier is a physical phenomenon with ammonia being bound under oxic conditions (see Hutchinson, 1957) or due to nitrification in aerobic surface sediments (see Larsen, 1977). Upon the development of anoxic conditions the physical state of the sediment could change, or nitrification becomes oxygen limited; either of these conditions will result in ammonia release from the sediment deposits. Possibly a combination of the two events may contribute to this process.

#### Nitrate

Prior to stratification nitrate, like ammonia, is evenly distributed throughout the depth profile, but at substantially higher concentrations ( $\sim 300 \mu\text{g N l}^{-1}$ ). Upon stratification nitrate assumes an inverse clinograde distribution due to rapid assimilation in surface water although nitrate does decrease in concentration, at slower rates, at other depths. This distribution becomes more clearly defined as the concentration of nitrate increases throughout the hypolimnion between June and August. This increase is concomitant with declining ammonia concentrations and therefore may be due to the activity of nitrifying bacteria. As oxygen becomes limiting, in the bottom waters, nitrate concentrations decline

leading to a dichotomic distribution (highest concentrations at intermediate depths) due to anaerobic nitrate reduction or denitrification in the profundal zone and assimilation in surface layers. Upon overturn nitrate becomes evenly distributed throughout the water column ( $\sim 200 \mu\text{g N l}^{-1}$ ) and concentrations increase to maximal values ( $\sim 650 \mu\text{g N l}^{-1}$ ) during the winter months. This maximum coincides with the minimum ammonia concentration. As the nitrate concentration within the lake, at this time, is greater than that in the main inflow it is probable that nitrification is responsible. Winter nitrate maxima have been reported for other lakes (Hutchinson, 1957).

In summary, assimilation is the dominant process in surface waters during the summer months. In the hypolimnion three distinct phases of biological nitrogen transformations can be observed in the sequence, ammonification, nitrification and denitrification or nitrate reduction. Similar sequences have been reported for Loch Balgavies (Christofi 1978) and for several Danish lakes (Larsen, 1977). In Loch Balgavies the cycles were repeated during the months following overturn. In the present study nitrification appeared to dominate in the winter months. Quantitative assessment of this activity is difficult due to the non-quantifiable nature of diffuse nitrate inputs into a freely mixed system. The data obtained from the metalimnion were difficult to interpret due to the availability of nitrogen species for the various biological transformations and enhanced bacterial activities of this depth layer caused by allochthonous inputs and increased temperatures. For convenience of interpretation depth distribution diagrams for both ammonia and nitrate are presented in Figure 5.3 for sampling dates which illustrate the cycles described above. Because of its relevance to the present work, and the following discussion, the nitrate increases in the depth layers 10 - 15 m and 15 - 20 m, for the years 1975, 76 and 77, are shown in Figure 5.4. Throughout the rest of this thesis these increases in nitrate

Figure 5.3 Depth distribution diagram for ammonia and nitrate concentration, in the water column of Grasmere, at specified dates which illustrate the overall seasonal cycles.

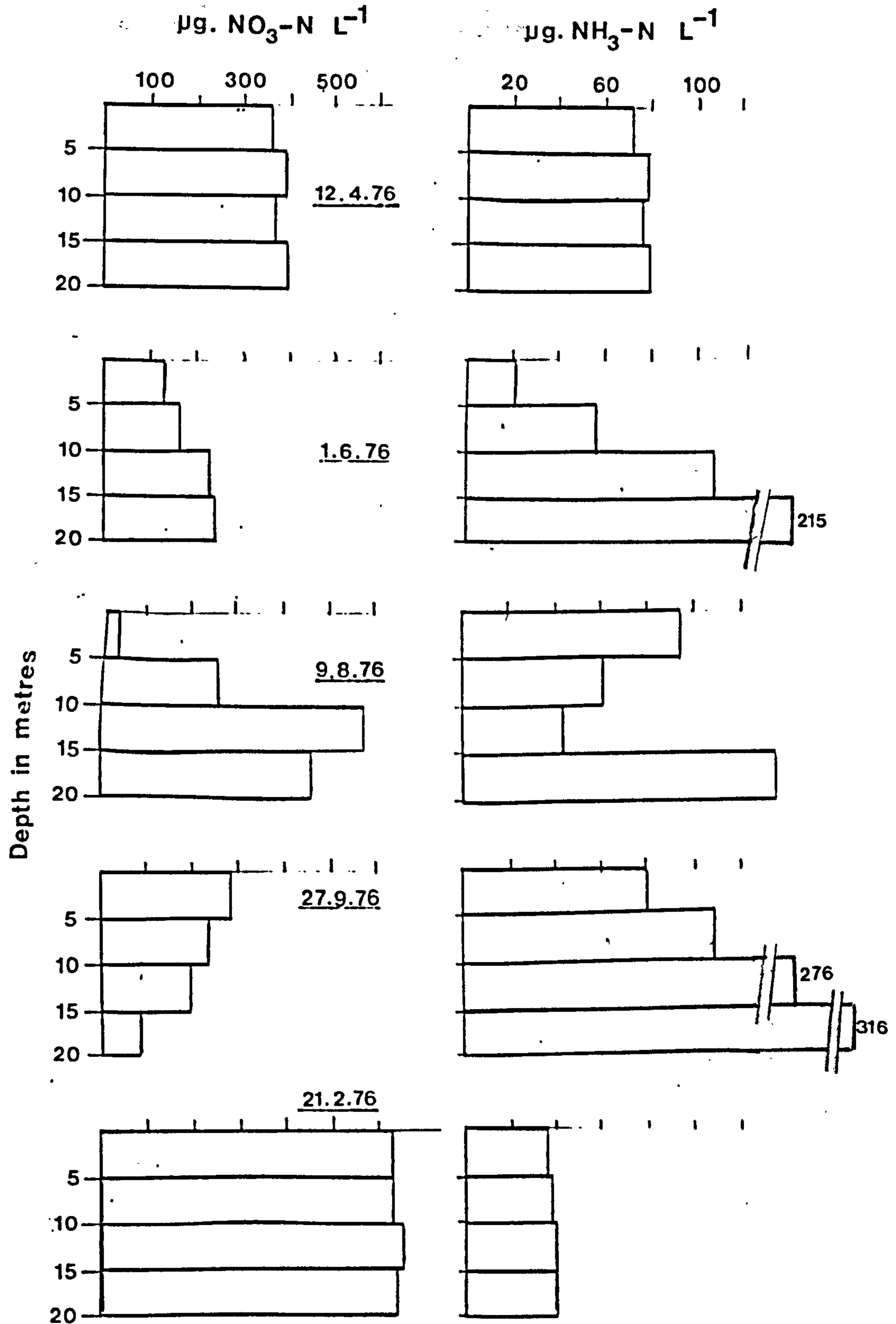
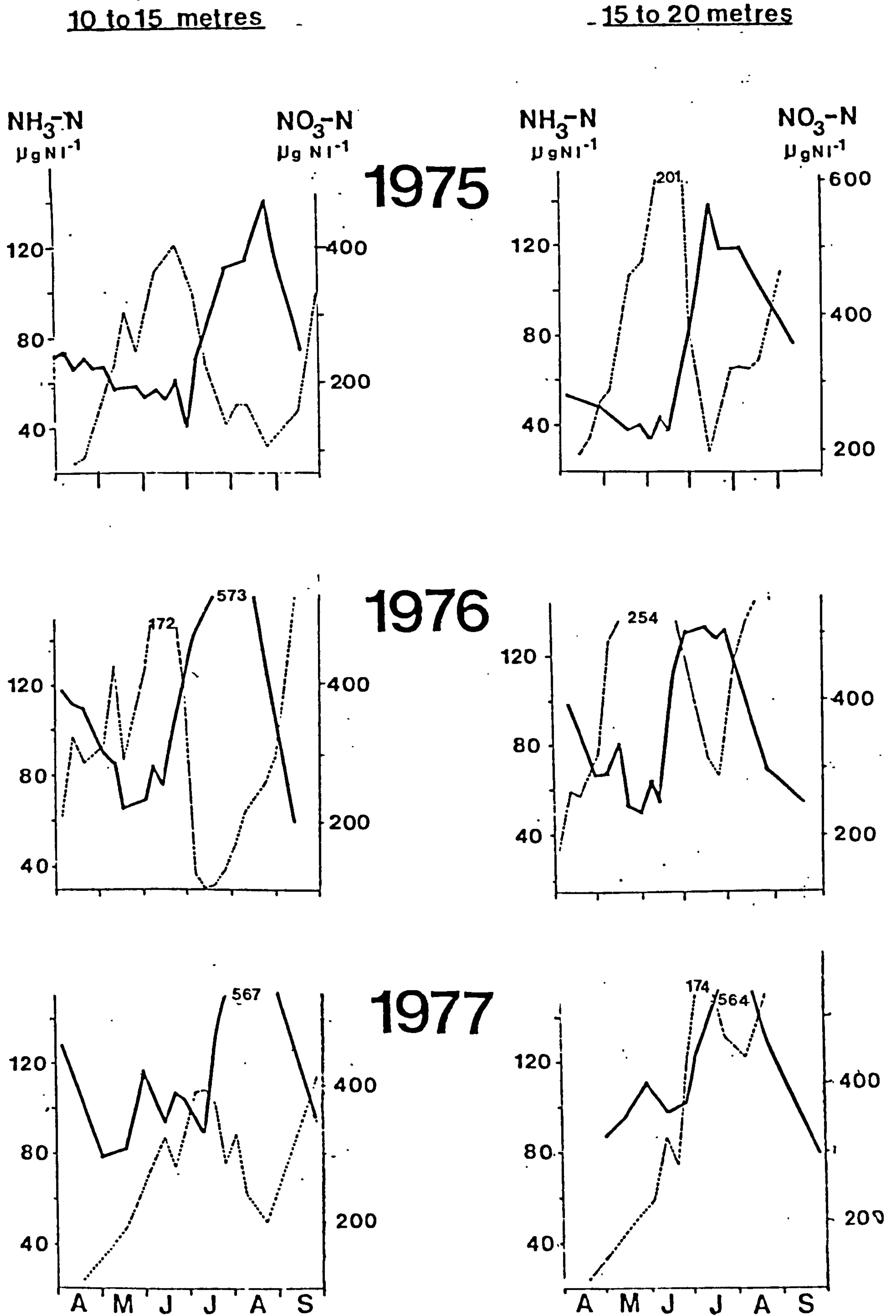


Figure 5.4 Variation, based on weekly measurements, in ammonia (---) and nitrate (—) concentrations over the period of stratification of Grasmere in 1975, 1976 and 1977, between the depth layers 10 - 15 m and 15 - 20 m showing the sequence of nitrogen cycle reactions ammonification, nitrification and denitrification.



concentrations, between June and July, will be referred to as the nitrification phase, as this is obviously the dominant process, and does not include increases in nitrate concentrations at other times of the year.

## 5.2 Observations during the nitrification phase in the hypolimnion

The nitrate ion is freely mobile between the sediments and the water column; the nett direction of movement being dependent upon the concentration gradient. Therefore the appearance of nitrate, in the deeper hypolimnetic layers is not a posteriori evidence for in situ nitrifying activity. In addition the increases in nitrate concentration were detected in pooled samples and it is not clear if the increases were due to activity at discrete depths influencing the whole depth layer chemistry. However, evidence for in situ activity is the simultaneous appearance of nitrate at 20, 18 and 16 m depths (see below). If release from sediments was responsible for the observed increases a moving gradient of nitrate into the water column would be expected. Excessive turbulent mixing could be eliminated because of the stability of the oxygen gradients at these depths.

Water samples from individual depths, taken during the nitrification phase and incubated under in situ conditions for three to five days continued to accumulate nitrate and, moreover, this accumulation was prevented by the addition of  $5 \mu\text{g ml}^{-1}$  N-Serve (in ethanol). Typical results are shown in Table 5.1. The control flasks received an equivalent amount of ethanol. This demonstrated that nitrification was occurring throughout the depth profile of the hypolimnion at site A and was mediated by planktonic chemolithotrophic nitrifiers. From the results of this experiment one cannot eliminate the possibility that methane oxidising bacteria participated in ammonia oxidation, but, for the purposes of the following discussion, it is not important which organisms are active

Table 5.1 Effect of  $5 \mu\text{g ml}^{-1}$  N-Serve on nitrate accumulation in laboratory incubated water samples taken during the nitrification phase (in June and July) in the hypolimnion of Grasmere.

Depth	Initial nitrate	Final nitrate	
		Untreated	$5 \mu\text{g. ml.}^{-1}$ N-Serve
10 metres	$371 \pm 12$ (11)	$422 \pm 19$ (14)	$370 \pm 9$ (7)
18 metres	$505 \pm 20$ (4)	$605 \pm 7$ (4)	$525 \pm 8$ (4)
17 metres	$591 \pm 22$ (4)	$659 \pm 7$ (4)	$550 \pm 6$ (4)
16 metres	$606 \pm 7$ (4)	$745 \pm 10$ (4)	$644 \pm 8$ (4)

all figures in  $\mu\text{g. NO}_3\text{-N l}^{-1} \pm 95\%$  c.l.

( ) degrees of freedom

since both possible types show similar stoichiometries for the oxidation of ammonia to nitrite (O'Neill and Wilkinson, 1977).

The relative importance of the nitrification reactions in deoxygenation of the hypolimnion can be assessed by calculation of the oxygen required to account for the observed nitrate concentration increases (during the nitrification phase) and comparing this to the total measured oxygen depletion of the hypolimnion throughout the period of stratification. Due to the differing volumes of the depth layers (10 - 15 m, 15 - 20 m) used in this study the calculation is more meaningfully expressed on a total volume basis (using the bathymetric data presented by Ramsbottom, 1976; Fig. 4.1) for the 15 - 20 m and 10 - 15 m depth layers. These whole lake calculations are valid as nitrate concentration increases were demonstrated, in the hypolimnion, at site B as shown in Figure 5.5. The values at site A are also shown for comparison. It appears that errors may occur in the calculation of total oxygen demand as the magnitude of the nitrate increase varies between the two sites. However, the calculations will give a general indication of the importance of the process.

The rate of total oxygen consumption was calculated from the difference in mean oxygen concentrations of the particular depth layer at the onset of stratification (taken as the date on which a difference of  $1^{\circ}\text{C}$  was established between 1 and 20 m depth) and at the sampling date prior to complete deoxygenation or the first observed depression of the thermocline, whichever came first. The nitrate increase was determined from field data (during the nitrification phase) and converted to oxygen values by using the theoretical oxygen to nitrogen ratio, for chemolithotrophic nitrifiers, presented by Wezernak and Gannon (1968). The results of these calculations are presented in Table 5.2 which shows that nitrification accounted for between 7 and 15% of the total oxygen consumption of the hypolimnion and was, therefore, a very important



Figure 5.5 Comparison of nitrate concentration increases during the nitrification phase of the hypolimnion of Grasmere, 1 m (—), 3 m (---) and 5 m (...) above the sediments at sampling sites A and B

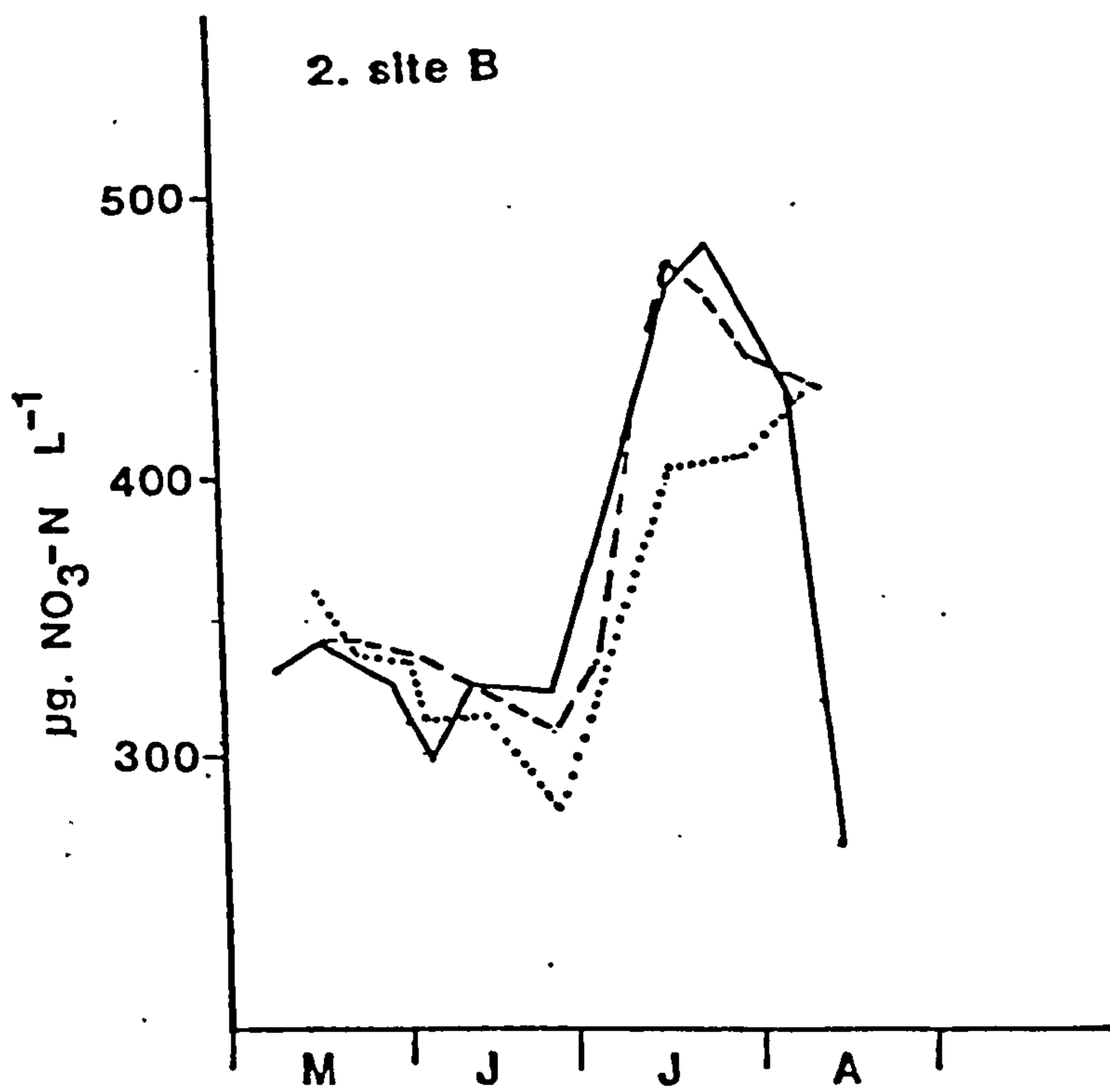
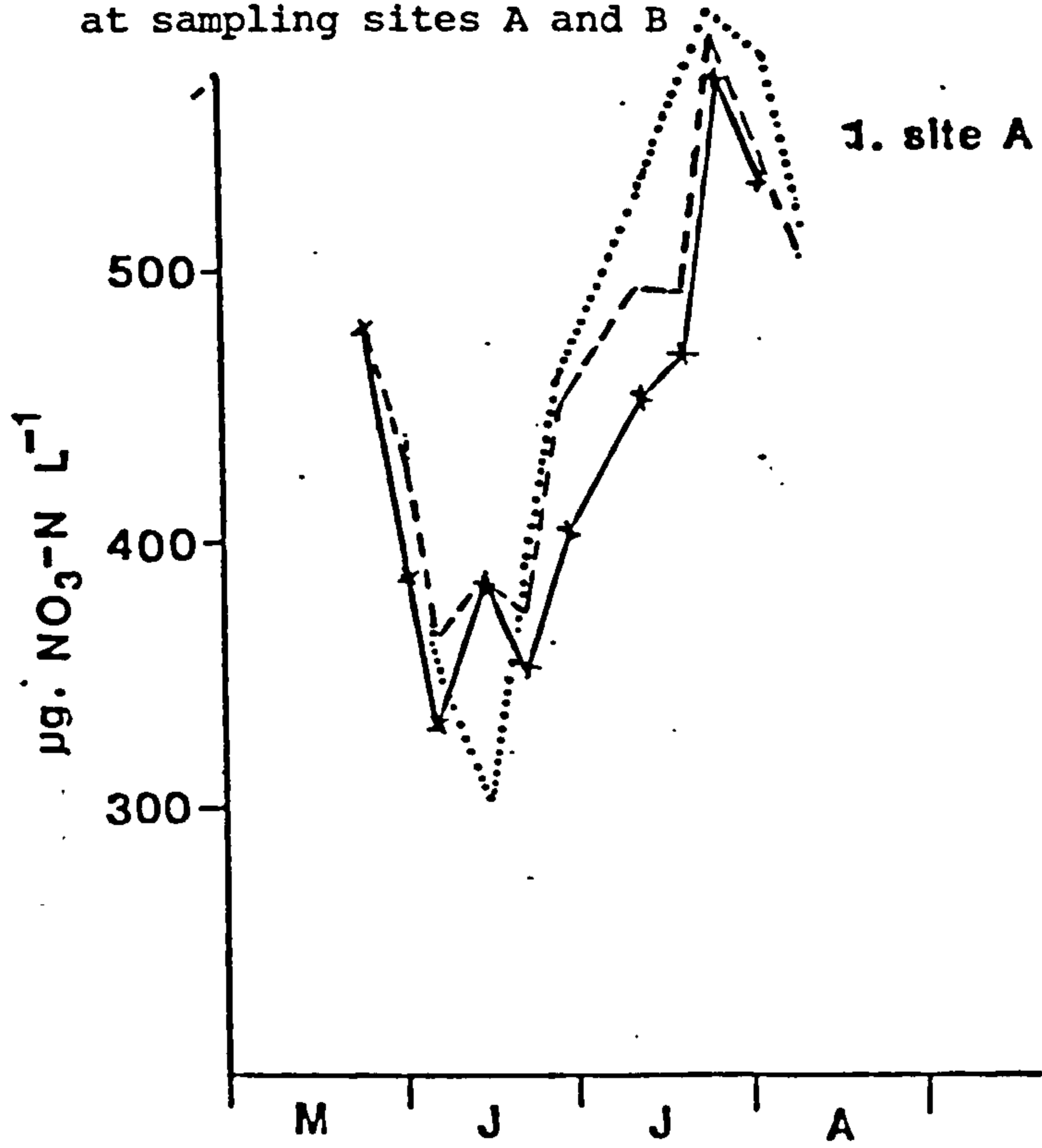


Table 5.2 The oxygen required to account for observed nitrate concentration increases during the nitrification phase of Grasmere, at the 10 - 15 m and 15 - 20 m depth layers, and its contribution to the total deoxygenation of these layers during stratification, over three years.

	1975		1976		1977	
	15 - 20m	10 - 15 m	15 - 20m	10 - 15 m	15 - 20m	10 - 20m
Total O <sub>2</sub> consumed	3.01 x 10 <sup>4</sup>	6.29 x 10 <sup>4</sup>	3.0 x 10 <sup>4</sup>	6.22 x 10 <sup>4</sup>	4.09 x 10 <sup>4</sup>	7.74 x 10 <sup>4</sup>
O <sub>2</sub> consumed by nitrate increase	0.4 x 10 <sup>4</sup>	0.9 x 10 <sup>4</sup>	0.35 x 10 <sup>4</sup>	0.85 x 10 <sup>4</sup>	0.3 x 10 <sup>4</sup>	0.77 x 10 <sup>4</sup>
Contribution	13.3%	14.3%	11.7%	13.7%	7.3%	10.0%

all units gO<sub>2</sub> depth layer<sup>-1</sup> day<sup>-1</sup>

process. These percentage values are lower than those presented by Hall et al (1978) as the present calculations were based only on the nitrification phase and not the difference in lowest and highest nitrate concentrations observed during summer stratification. It should be noted that these figures are minimal values as gross nitrate production in the water column could be greater due to nitrate utilization. In addition no account was taken of nitrifying activity in the sediments or water column prior to the nitrification phase.

Chemolithotrophic nitrification should result in a ratio of ammonia loss to nitrate gain of unity. For the three years data presented this ratio varies between 0.3 and 0.7. As pointed out by Hall et al (1978) there is no reason to assume that the ammonification processes, preceding the nitrification phase, have stopped. The gross ammonia change is the sum of the two rates. Using this correction the ratio varies between 0.3 and 1.33. However, a similar assumption must be made for nitrate, which was declining prior to the nitrification phase, and again the gross rate of nitrate increase is the sum of the two rates. Using this correction the ratio varies between 0.3 and 0.88 indicating a negative balance for ammonia. The difference must be supplied by active nitrogen mineralization. These calculations emphasize the inaccuracies which can result in the estimation of rates of bacterial activity from the rates of change in net nutrient concentrations observed in the field.

It is usual to observe a peak in nitrite concentration, in a system in which chemolithotrophic nitrifiers are becoming active, as this is the required substrate for phase II organisms, which have to be induced. Nitrite was never detected in significant concentrations and therefore either the sampling interval of 7 days was too long to detect transient peaks or the population was already established and ready to respond to changes in the chemical environment.

If the first observed increase in nitrate concentration is taken as the beginning of the nitrification phase, activity within the 15 - 20 m depth layer commences approximately 14 days earlier than in the 10 - 15 m depth layer (Figure 5.4). Activity also persists in the 10 - 15 m layer for between 14 to 19 days longer than in the deeper hypolimnion. It has already been demonstrated (Fig. 5.5) that simultaneous nitrate increases occur at depths of 20, 18 and 16 m so larger depth intervals have to be investigated to show this 'moving front' effect, possibly caused by oxygen depletion moving up the water column.

The inter-relationship of the nitrification phase and other chemical parameters is worthy of further consideration. Over the three years study period the ammonia concentration, at the start of the nitrification phase, varied between 100 and 254  $\mu\text{g N l}^{-1}$ . There does not appear to be a critical concentration at which the nitrification process becomes dominant. The concentration at the 15 - 20 m depth layer was always greater than that at the 10 - 15 m layer. Also, once activity had commenced the ammonia concentration declined to very low levels (0 to 60  $\mu\text{g N l}^{-1}$ ). The oxygen concentrations, at the onset of the nitrification phase varied between 7.4 and 9.5  $\text{mg O}_2 \text{l}^{-1}$  for the 10 - 15 m layer, and 7.8 to 6.05  $\text{mg O}_2 \text{l}^{-1}$  for the 15 - 20 m layer. For each particular year the oxygen concentration was greater in the 10 - 15 m layer than in the lower depths. This type of relationship would be expected as there is an oxygen consumption gradient into the water column due to the greater metabolic activity in the sediments. Interestingly, loss of nitrate from the water column started at oxygen concentrations of 0.3 to 2.3  $\text{mg O}_2 \text{l}^{-1}$ . This indicates that anaerobic processes were theoretically active in a chemically oxidised environment, but does not preclude the possibility of anaerobic microsite development within particulate material.

### 5.3 Nitrification rates in the hypolimnion

The occurrence of planktonic nitrification in the hypolimnion has already been demonstrated and calculations, based on nitrate concentration increases during the nitrification phase, and reported in the previous section, show this to be a significant oxygen sink. The importance of this process could, however, have been underestimated partly due to the possibility of nitrification occurring prior to the increases in nitrate concentration in June and July. Therefore attempts were made to estimate rates of nitrification, at discrete depth intervals, throughout the period of stratification.

#### 5.3.1 General

Attempts to measure in situ rates of bacterial activity involve the incubation of enclosed water samples. In order to obtain realistic measurements the total bacterial population of the sample must not change significantly from conditions in situ. This bottle effect, discussed on page 43, was examined, using epifluorescent direct bacterial counts, on lakewater samples incubated at the average temperature of the hypolimnion, 10°C. These results, along with many others obtained in this laboratory over the years, are shown in Figure 5.6 (from Jones, 1977). Generally incubation periods of greater than 48 hours should be avoided at this temperature.

As discussed in Chapter 2, the best available methods for assessing nitrification in situ involve the use of nitrification inhibitors. These sample additions (in this case an alcoholic solution of N-Serve) could affect the total bacterial population. This was tested using filter sterilized N-Serve (in ethanol) solutions (0.22  $\mu$  Nucleopore filters) and epifluorescent counts of the bacteria. Typical results obtained after 48 hours incubation are shown in Table 5.3. Both the N-Serve solution and ethanol alone cause significant increases over control values.

Figure 5.6 Effect of temperature and time on total bacterial counts in stored water samples (from Jones 1977)

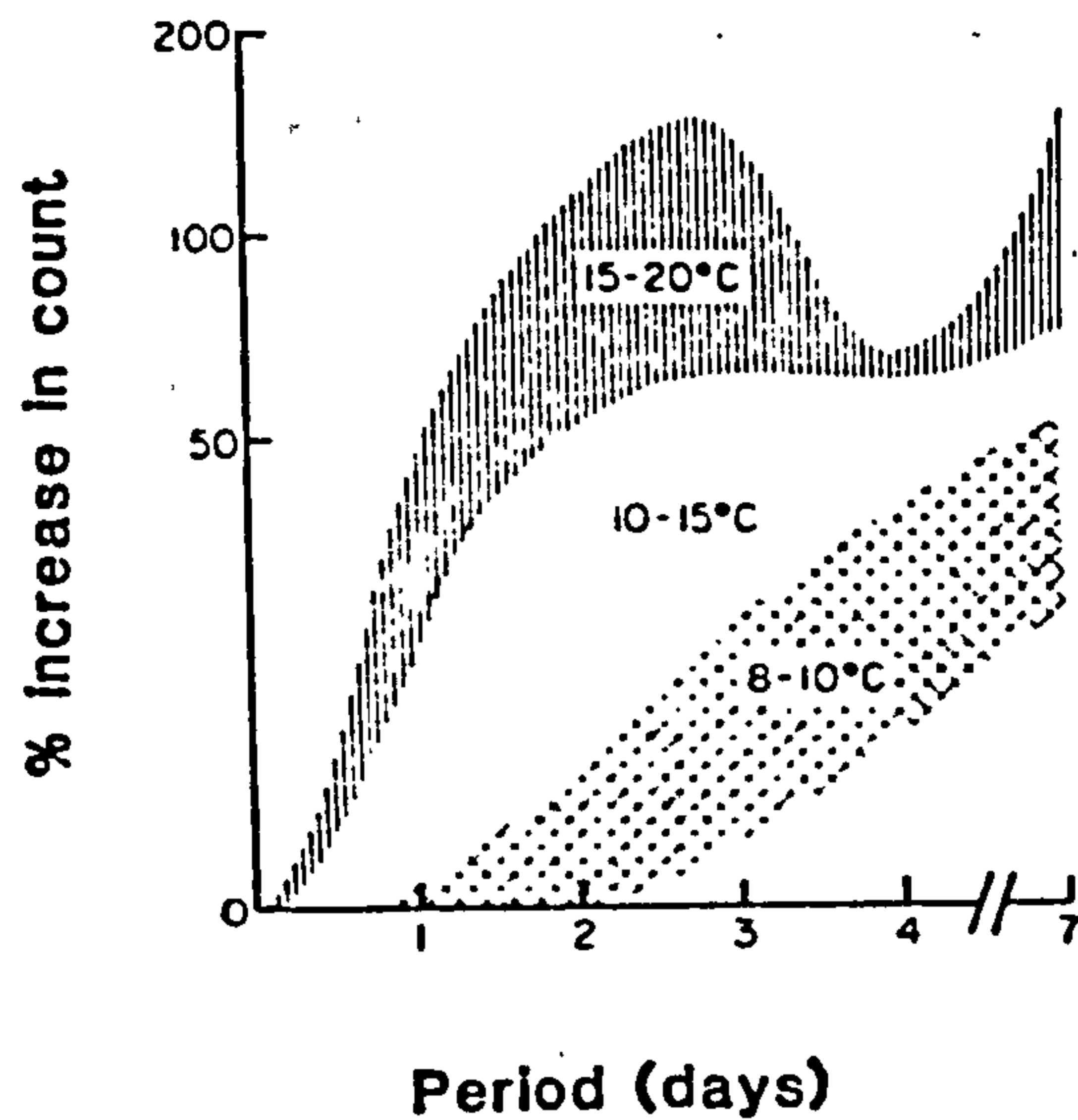


Table 5.3 Effect of  $10 \mu\text{g ml}^{-1}$  N-Serve and  $100 \mu\text{g ml}^{-1}$  ethanol additions on the total bacterial populations of water samples (135 ml) incubated for 48 hrs at  $10^{\circ}\text{C}$ .

	CONTROL	N-SERVE	ETHANOL
Time zero	1.33 <sup>b</sup>	1.33	1.33
Time 48 h	2.82	3.57	3.61
Time zero	1.31	1.31	1.31
Time 48 h	2.37	3.11	3.22

<sup>b</sup> all counts as numbers of bacteria  $\times 10^6 \text{ ml}^{-1}$

95% confidence limits are  $\pm 10\%$  of the count

This is probably a direct effect of the ethanol which can act as a carbon and energy source for many bacteria. These results indicate that incubation times of 48 hours are not advisable and for valid results considerably shorter times are required.

The increases in bacterial populations, with time, are often discussed in relation to substrate concentrations and increased bacterial growth at the solid-liquid interface. This suggests that samples from different environments, and the use of different sample bottle sizes, will affect the results obtained. The results in Figure 5.6 are presented as percentage increases in an attempt to draw some general conclusions. Other workers estimating nitrification rates have not investigated this phenomenon and therefore it is impossible to compare findings.

### 5.3.2 Choice of method

Having established that, due to the natural bottle effect and the stimulation of this by sample additions, 24 h was the maximum incubation time allowed for estimation of nitrification rates, an assessment of the possible usefulness of methods (Section 2.3.3) can be made.

The maximum rate of increase in nitrate concentration observed within the lake during 3 years investigation of the hypolimnetic nitrification phase, was  $7 \mu\text{g NO}_3\text{-N l}^{-1} \text{ day}^{-1}$ . Although this increase was not a reliable index of in situ activity it provides an indication of the level of expected differences to be found between inhibitor treated and untreated samples. Knowing that the actual nitrate concentrations in the samples varied between 300 and  $500 \mu\text{g N l}^{-1}$  it can be calculated, from within sample variation of the nitrate determination, that between four and six replicate determinations would be required, per sample, to detect a difference between means of  $10 \mu\text{g NO}_3\text{-N l}^{-1}$  ( $P > 0.05$ ; J. Hilton pers. comm.). Assuming that both inhibitor and control treatments were duplicated, and four depths were sampled, a total of 64 to 96 nitrate

determinations would have to be carried out; the number increases with nitrate concentration. This would be impractical on a routine basis. A similar precision could be expected of a continuous flow auto-analyser system (J. Hilton pers. comm.) and therefore with the elimination of manual procedures the method does become possible. However, auto-analyser facilities were not available at the time of study. The ammonia determination described has been shown to have a smaller within sample variation, but, due to the small changes in ammonia concentration noted within the lake it is likely to be of little use.

The Winkler method for oxygen determination has been extensively used in this laboratory over a number of years and the precision of its determination is well established. Using five replicate bottles, at an oxygen concentration of  $10.5 \text{ mg l}^{-1}$ , a standard deviation of 0.17% was obtained (J.G. Jones pers. comm.). The 95% confidence limits of this determination would therefore be  $\pm 0.022 \text{ mg O}_2 \text{ l}^{-1}$  (4 degrees of freedom). Again using  $10 \text{ } \mu\text{g NO}_3\text{-N l}^{-1}$  as a maximum difference expected, this corresponds to an oxygen difference of  $45.7 \text{ } \mu\text{g O}_2 \text{ l}^{-1}$ . Therefore the between sample variation accounts for approximately 50% of the estimated maximum difference and so the method would only be of minimal use for water samples. The between sample variation is unlikely to increase with decreasing total oxygen concentration until values of less than  $0.5 \text{ mg l}^{-1}$  are obtained (J.G. Jones pers. comm.).

No information was available concerning the variability of dark  $^{14}\text{CO}_2$  uptake estimations but the sensitivity of radiotracer techniques is well established. Billen (1976) reported results, using the  $^{14}\text{C}$ -bicarbonate and N-Serve method (described in section 4.7 ) to be within an order of magnitude of the actual nitrification rates occurring in marine sediments. Christofi (1978) reported excellent agreement between nitrification rates, estimated using this method, and observed nitrate increases in the hypolimnion of Blelham Tarn. Recently Fenchel and



Blackburn (1979) stated that this method showed great promise for estimating in situ nitrification rates and this was the method adopted throughout the present study.

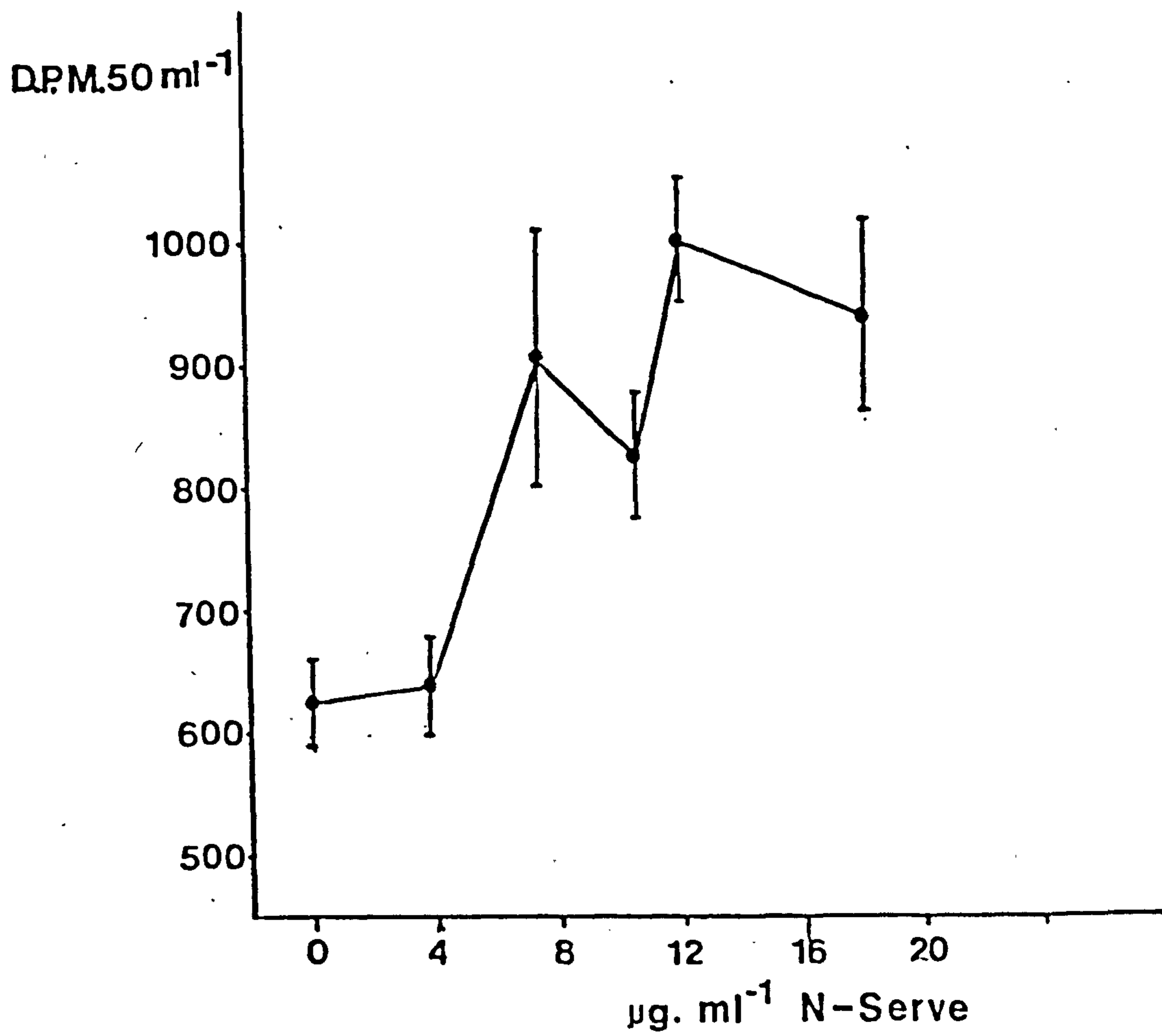
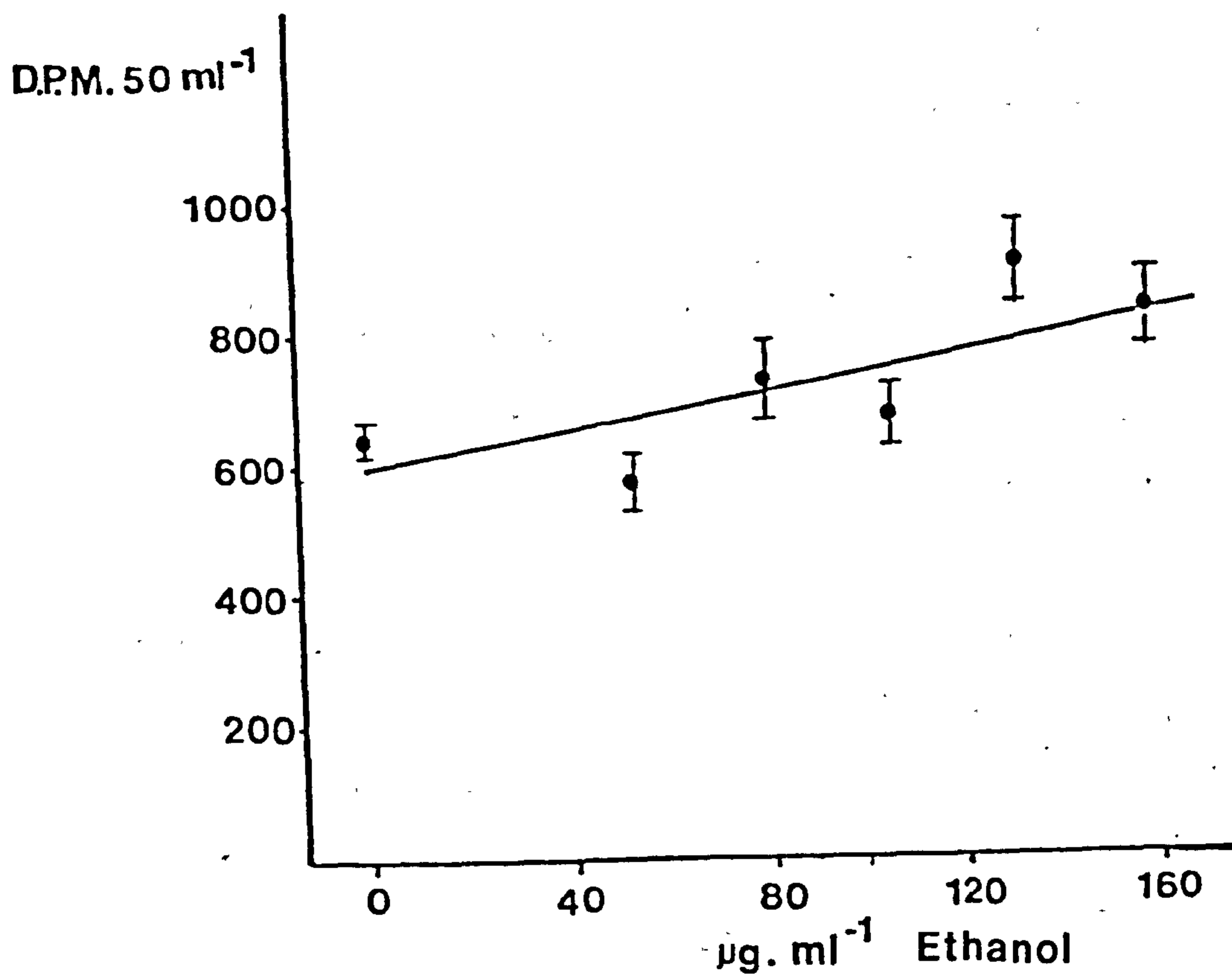
### 5.3.3 Validation of method

It has already been emphasized that additions to the water sample ideally should have no effect on the total bacterial population or the particular biological activity being investigated; in this case the dark carbon dioxide uptake (hereafter referred to as CO<sub>2</sub> uptake). The effect of increasing concentrations of ethanol on the CO<sub>2</sub> uptake of both natural and enriched bacterial populations was investigated. The samples were thoroughly mixed and equal volumes dispensed into sterile 135 ml bottles. Ethanol and approximately 3 µCi. (111 k.Bq.) of <sup>14</sup>C-bicarbonate were added to each bottle. Six replicates per ethanol concentration were prepared. Typical results are shown in Figure 5.7 where the carbon uptake values are expressed as disintegrations per minute per unit volume. For statistical analysis a logarithmic data transformation was used. Increasing the concentration of ethanol stimulated the CO<sub>2</sub> uptake and therefore the lowest concentration of ethanol used (53 µg ml<sup>-1</sup>) was subsequently adopted as the maximum concentration which could be added to samples for nitrification rate estimation. The effect of N-Serve concentration on dark CO<sub>2</sub> uptake was tested in a similar manner with a final ethanol concentration of 53 µg ml<sup>-1</sup> used in all cases. Typical results are shown in Figure 5.8 and a marked stimulation of dark CO<sub>2</sub> uptake was observed between 4 and 12 µg ml<sup>-1</sup> N-Serve. Subsequent experiments showed this stimulation to occur at 7 µg ml<sup>-1</sup> N-Serve and therefore the concentration of 5 µg ml<sup>-1</sup> as used by Billen, 1976; Somville, 1978 and Christofi, 1978 was used as the maximum concentration to be added to samples.

Two problems were associated with the maximum allowable ethanol and N-Serve concentrations found from the above experiments. Firstly organic

Figure 5.7 Effect of ethanol concentration on the dark CO<sub>2</sub> uptake of stored water samples incubated for 24 hours at in situ temperature (10°C). Results are expressed as d.p.m. as all factors for total CO<sub>2</sub> uptake calculation were constant.

Figure 5.8 Effect of N-Serve concentration on the dark CO<sub>2</sub> uptake of stored water samples incubated for 24 hours at in situ temperature (10°C). Final ethanol concentration was 53 µg ml<sup>-1</sup> in all cases. Results are expressed as d.p.m. as all factors for total CO<sub>2</sub> uptake calculation were constant.



solvents had been reported to inhibit certain bacterial nitrogen transformations (Bremner et al, 1979) and therefore this effect should be investigated on the nitrification process. In addition, when the N-Serve solution is added in the specified concentrations, to water samples, some N-Serve comes out of solution due to the absence of sufficient carrier solvent. It was therefore necessary to determine if the N-Serve in solution was still inhibitory to nitrification. This was investigated using an enrichment of chemolithotrophic nitrifiers prepared by adding  $(\text{NH}_4)_2\text{SO}_4$  to lake water and aerating. The results are shown in Figure 5.9 and it is apparent that  $53 \mu\text{g ml}^{-1}$  ethanol had no effect on the nitrification process whilst  $5 \mu\text{g ml}^{-1}$  N-Serve, under these conditions, remained inhibitory for up to 19 hours. This is approximately the maximum incubation time which can be used for nitrification rate estimates. Further investigation indicated that these concentrations had no effect on the total bacterial populations of stored water samples as shown in Table 5.4.

#### Discussion

The reason for the stimulation of microbial activity, by concentrations of N-Serve greater than  $7 \mu\text{g ml}^{-1}$ , is, as yet, unknown. Some organic herbicides have also been shown to have this effect (Weeraratna, 1979). The efficiency of N-Serve to inhibit nitrification, is known to be decreased in the presence of organic matter (Goring, 1962). The nitrifier enrichment, used in the above experiment would be likely to contain more organic material than natural water samples and so some confidence could be placed in the  $5 \mu\text{g ml}^{-1}$  N-Serve remaining inhibitory for the 24 h duration of subsequent sample incubation periods. Bremner et al (1979) state that the organic carrier solvents used for N-Serve formulations can affect nitrogen transformations by soil microorganisms and therefore aqueous solutions, prepared by shaking the compound, with water, in a

Fig. 5.9 Effect of  $53 \mu\text{g ml}^{-1}$  ethanol and  $5 \mu\text{g ml}^{-1}$  N-Serve on nitrate production by an enrichment culture of lithotrophic nitrifying organisms.

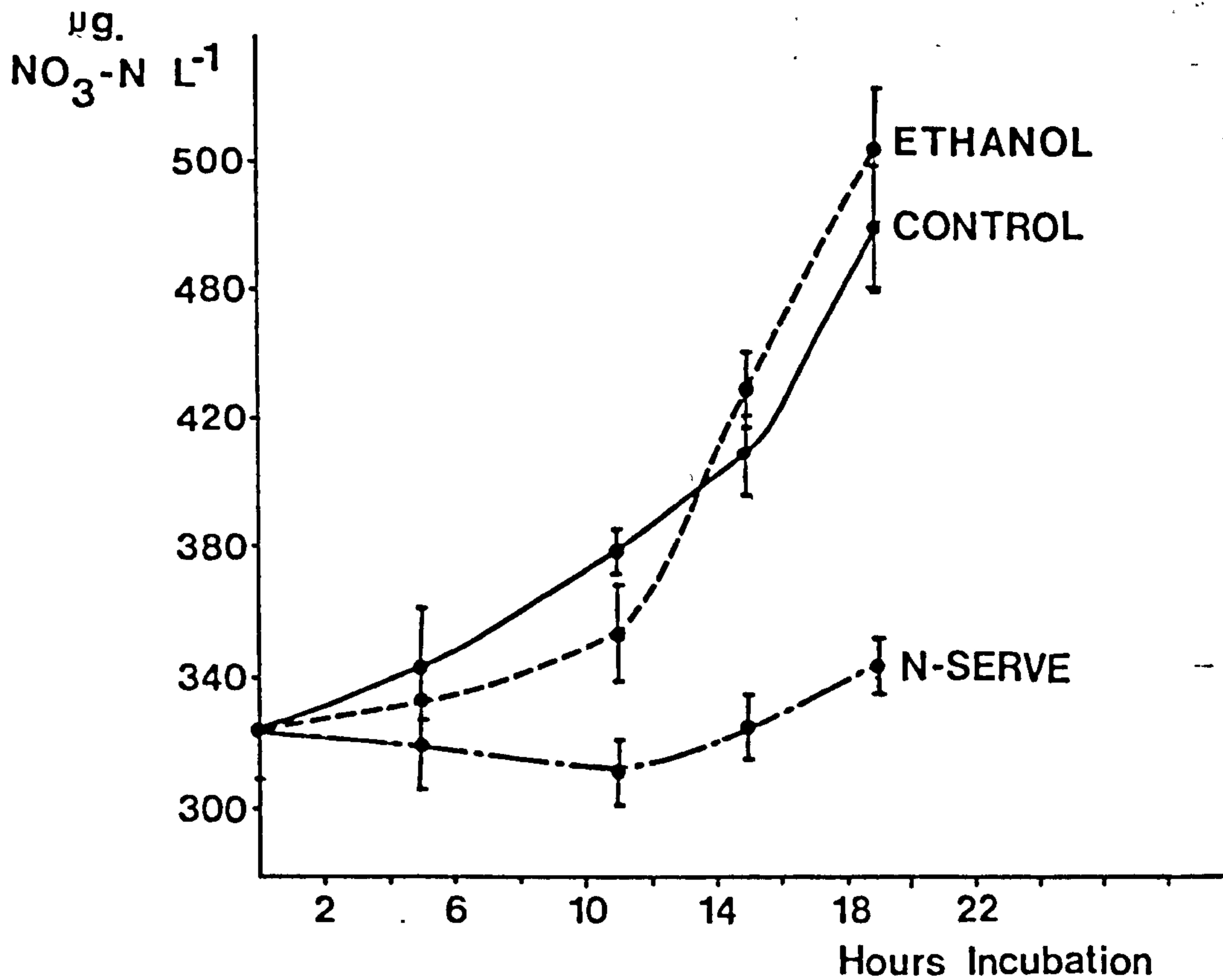


Table 5.4 Effect of  $5 \mu\text{g ml}^{-1}$  N-Serve and  $53 \mu\text{g ml}^{-1}$  alcohol on the total bacterial populations of water samples (135 ml) incubated for 24 hours at  $10^{\circ}\text{C}$ .

	CONTROL	N-SERVE	ALCOHOL
Time zero	$1.17^{\text{b}}$	1.17	1.17
Time 24 h	1.11	1.08	1.05
Time zero	1.08	1.08	1.08
Time 24 h	1.16	1.04	1.04

<sup>b</sup> all counts as numbers of bacteria  $\times 10^6 \text{ ml}^{-1}$   
 95% confidence limits are  $\pm 10\%$  of the count

stoppered bottle for 16 to 24 hours, should be used. Goring (1962) reports the solubility of N-Serve, in water at 22°C, to be 40 mg l<sup>-1</sup>. Therefore, assuming a saturated aqueous solution, 16.9 ml of inhibitor solution would have to be added to a 135 ml sample to obtain a final N-Serve concentration of 5 µg ml<sup>-1</sup>. This represents a sample dilution of almost 13% (V/V) and would affect natural nutrient concentrations substantially. N-Serve solubility in ethanol is reported to be 290 g.kg<sup>-1</sup> of solvent which is equivalent to 367 g.l<sup>-1</sup> (assuming a specific gravity of 0.79). Bremner et al (1979) do not report the concentrations at which organic solvents become inhibitory, or the specific nitrogen transformations which are susceptible. The present results indicate that ethanol, at the concentration used (53 µg ml<sup>-1</sup>) has no effect on chemolithotrophic nitrification. It is unfortunate that this work was not extended to investigate the effects of increased ethanol concentrations. Somville (1978) used a final ethanol concentration of 226 µg ml<sup>-1</sup> and Christofi (1978) used 6.3 mg ml<sup>-1</sup>. It is not possible to speculate as to the inhibitory action of these concentrations on nitrification. The present results indicate that these concentrations would greatly stimulate the dark CO<sub>2</sub> uptake and the values would, therefore, bear little relation to in situ activity.

It is interesting to note that of all the investigations, in the aquatic environment, which make use of the inhibitor N-Serve (Webb and Wiebe, 1975; Vanderborght and Billen, 1975; Cavari, 1977; Isirimah et al, 1976; Miyazaki et al, 1975; Billen, 1976; Chen et al, 1972a; Indrebo et al, 1979; Christofi, 1978; Somville, 1978) only the latter two give sufficient information to make the above calculations. The majority of reports only give details of the final inhibitor concentration and no solvent is mentioned. Of the original reports on the use of N-Serve. Goring (1962) used acetone as the solvent whilst Campbell and Aleem (1965) use alcohol, stock solution concentrations are not given. The most frequently cited

report on the specificity of N-Serve (Shattuck and Alexander, 1963) makes no reference to carrier solvents.

The final concentrations of  $53 \mu\text{g ml}^{-1}$  ethanol and  $5 \mu\text{g ml}^{-1}$  N-Serve, in all water samples, was maintained throughout this study. Unfortunately this protocol could not be followed during the studies on sediment samples (see later). The inhibitor was added to a final concentration of  $5 \mu\text{g ml}^{-1}$  which resulted in a final ethanol concentration of 2.0 to  $2.6 \text{ mg ml}^{-1}$  sediment (10  $\mu\text{l}$  inhibitor solution added to 3 or 4 ml sediment volume). The effect of  $5 \mu\text{g ml}^{-1}$  N-Serve on nitrification in sediments was not tested. Christofi (1978) found that  $1 \mu\text{g ml}^{-1}$  N-Serve inhibited nitrate production by Loch Balgavies sediment slurries (which had been diluted by 50% with overlying water), for up to 7 days. N-Serve has also been shown to inhibit nitrification in the sediments of other lakes (Chen et al, 1972a; Isirimah et al, 1976), therefore the chemolithotrophs appear to be the dominant nitrifiers in freshwater sediments.

#### 5.3.4 Nitrification rate estimates in the hypolimnion

The hypolimnion of Grasmere, for the purposes of this investigation, has been defined as water of greater than 10 m depth. Water samples were taken, for the estimation of nitrification rates, at 1 m depths, and weekly time intervals, from May to August, 1977. The 20, 18, 16, 14, 12 and 10 m samples were taken on one day and the remaining depths two days later. The N-Serve sensitive  $\text{CO}_2$  uptake was calculated only when there were significant differences between the untreated and N-Serve treated  $\text{CO}_2$  uptake values. It should be noted that replicate samples from each depth were not studied. Therefore, although parametric tests were used for data analysis it is not known if the assumptions, for these tests to be valid, are true for these data. Where significant differences were detected ( $P > 0.05$ ) the N-Serve sensitive  $\text{CO}_2$  uptake was converted to

the rate per day and multiplied by 9.01 to obtain the rate of nitrate production (see section

In order to compare these measured rates with the nitrate data for the depth strata, 10 - 15 m and 15 - 20 m, the mean rates between these depths were calculated. These are compared with the nitrate data in Figure 5.10; the nitrification phase (based on consistent nitrate concentration increases) is also indicated. It can be observed that each depth stratum exhibits peaks of activity, in May and July. The July peak at 15 - 20 m preceding the one at 10 - 15 m by approximately three weeks. The highest individual estimates of nitrification rate are measured in July ( $7 \mu\text{g N l}^{-1} \text{ day}^{-1}$ ), during the nitrification phase. However, high estimated nitrification rates are not found throughout the whole nitrification phase. The reasons for this are unclear as increases in field nitrate concentrations, at this time of year, have been shown to be inhibited by N-Serve (Table 5.1). The peak of nitrifying activity in May indicates that the calculations establishing the contribution of nitrification to the oxygen deficit of the hypolimnion (Table 5.2) will be underestimates as these were only based on nitrate concentration increases in June and July (the nitrification phase). In an attempt to calculate the full contribution of nitrification to the hypolimnetic oxygen deficit the estimated nitrification rates were used to calculate the cumulative total of nitrate production over the whole sampling period. To do this the regression line of the overall field nitrate data, on time, was calculated. The intercept of this line was taken as the initial nitrate value and the cumulative total was calculated from the sum of this and the average rate of nitrate production, for each depth layer, over each week. The assumption was made that the measured rate was sustained until the next sampling date. Each sum was used as the initial nitrate concentration for the following week. A regression line was then calculated for these data. The results are shown in Figure 5.11



Figure 5.10 The estimated nitrification rates (o) in  $\mu\text{g N l}^{-1} \text{ day}^{-1}$  and nitrate concentrations (●)  $\mu\text{g N l}^{-1}$  between 10 - 15 m and 15 - 20 m in Grasmere between May and August 1977. The nitrification phase (based on consistant nitrate concentration increases) is also indicated.

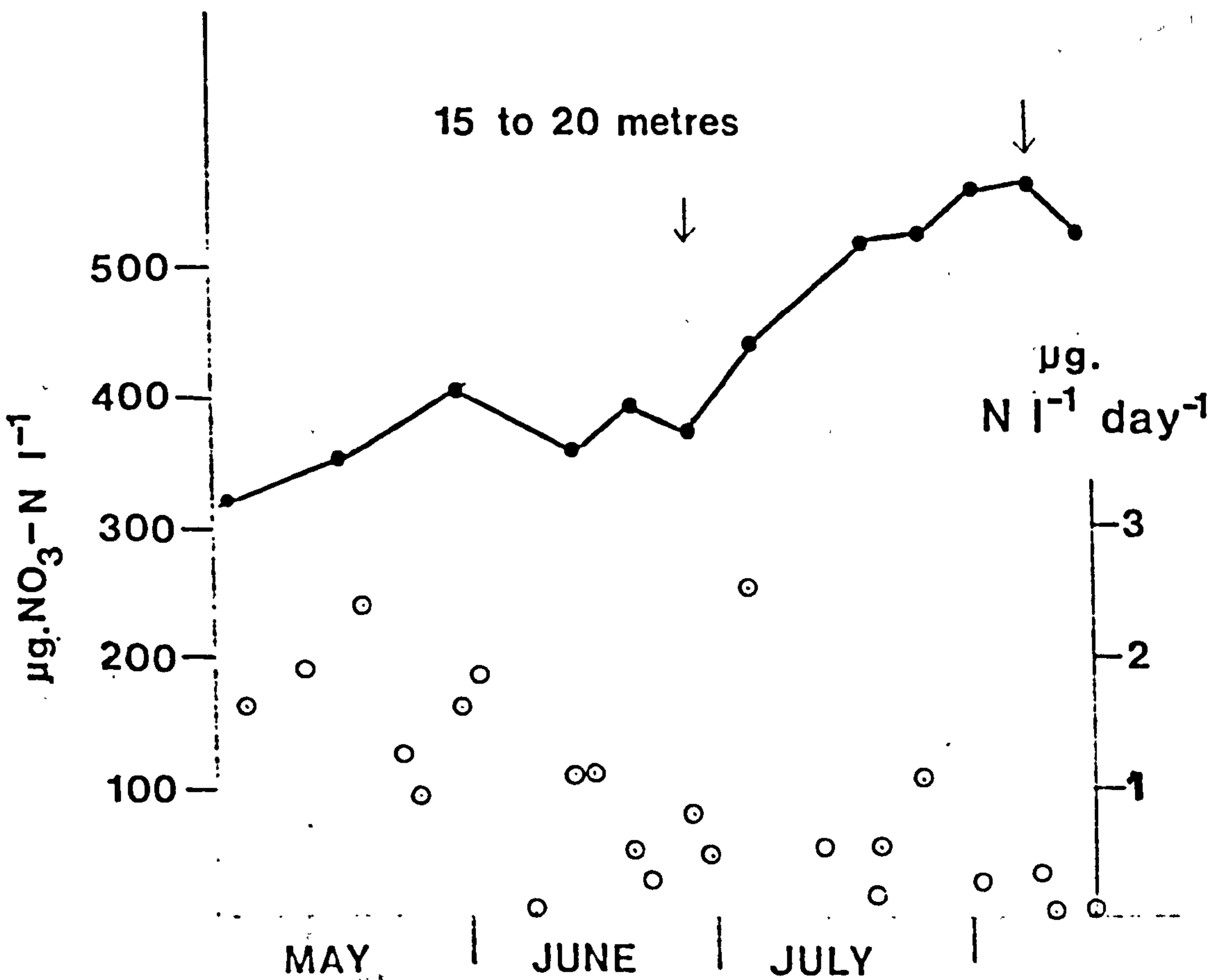
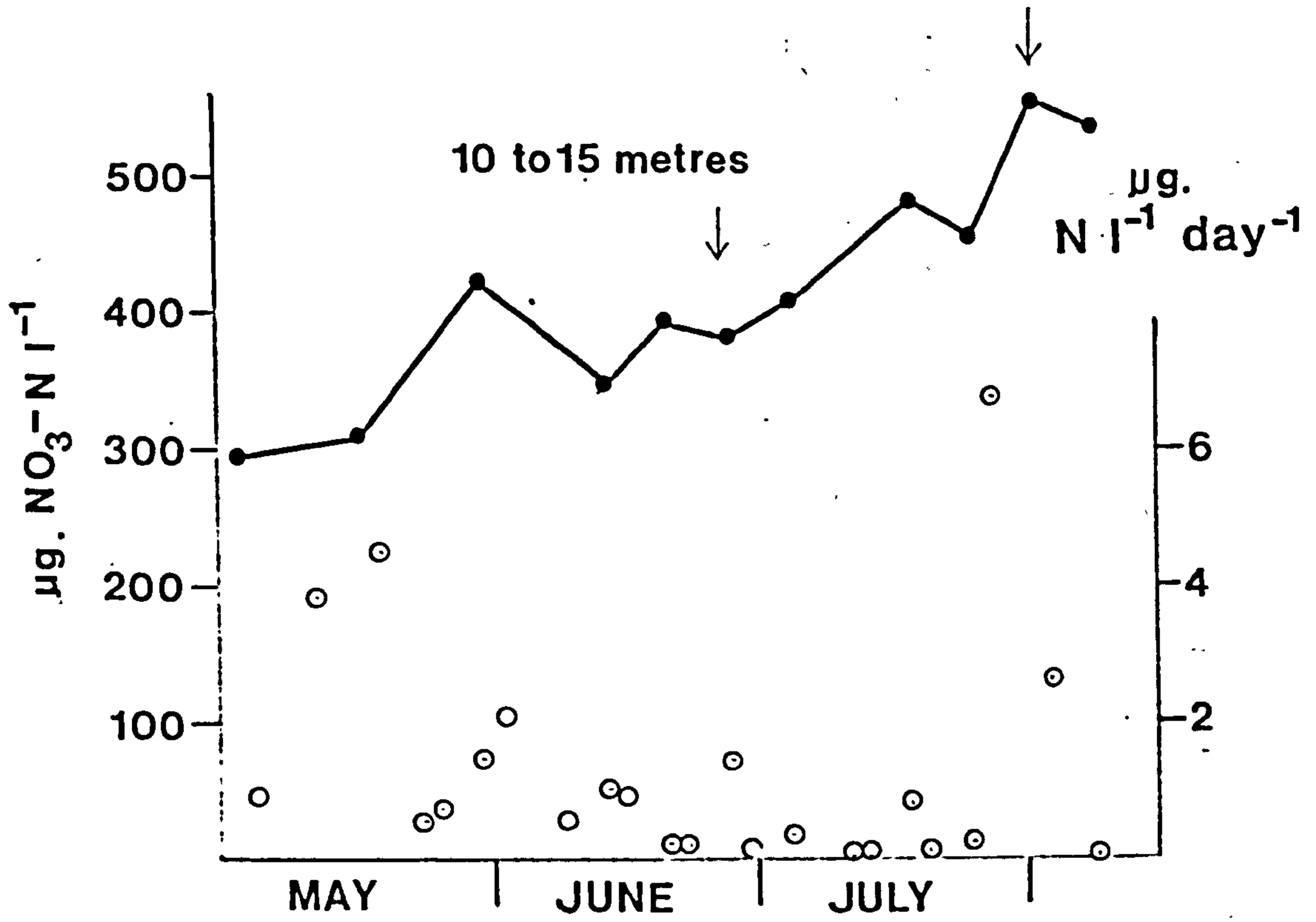
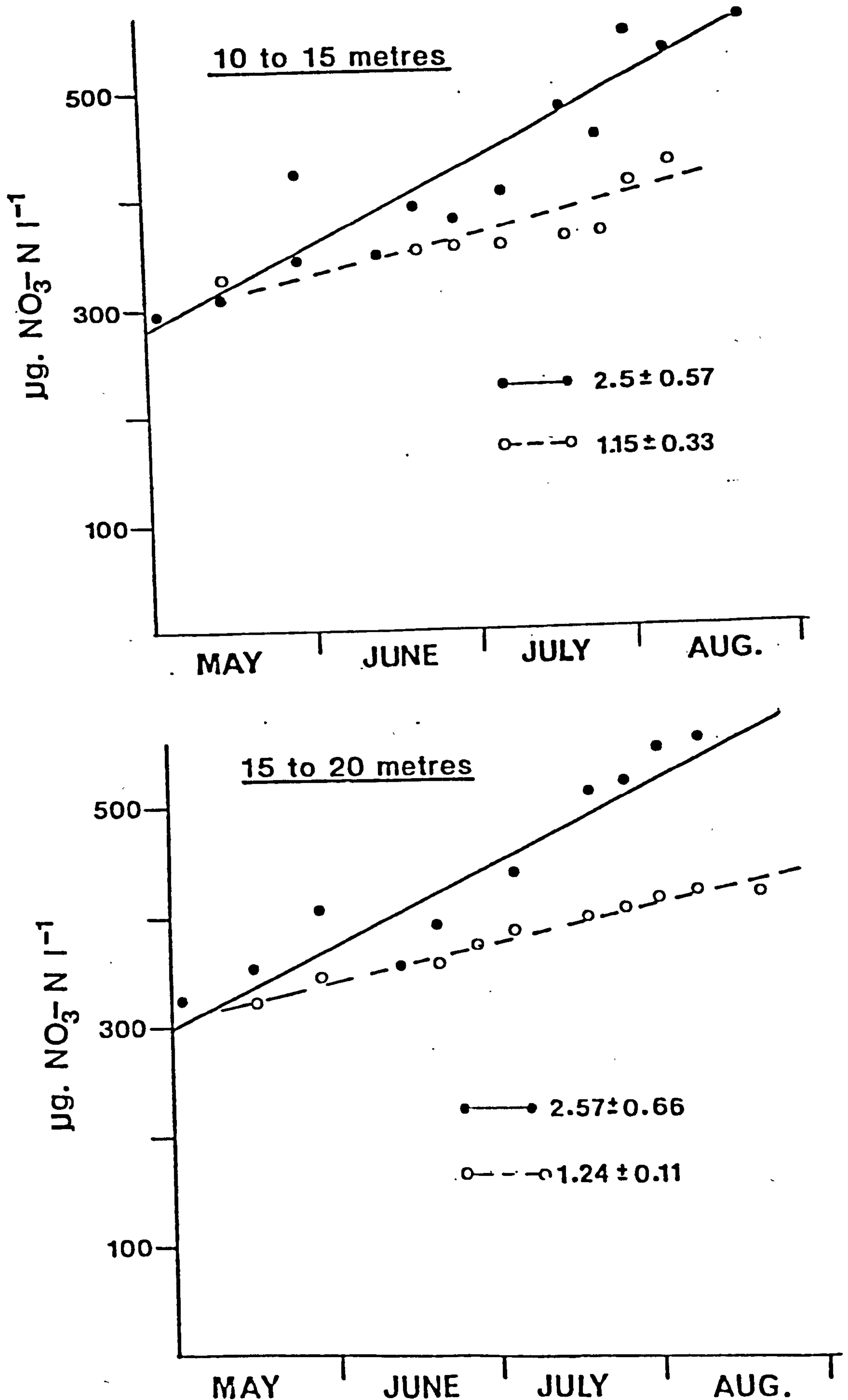


Figure 5.11 Comparison of regression lines of field nitrate concentrations (●) and cumulative nitrate concentration (○) (calculated from estimates of the nitrification rate), both on time in the 10 - 15 m and 15 - 20 m depth layers of Grasmere 1977, throughout the period of stratification. The slopes and 95% confidence limits of each line are also shown. The intercept of the regression line for field nitrate concentrations was used as the initial nitrate concentration for the estimated cumulative total.



and include the slope for each line and its corresponding 95% confidence limits. Although the full analysis is not shown the slopes of the two lines, at each depth layer, are significantly different ( $P > 0.05$ ) and therefore the method, as used, appears to underestimate the actual nitrification rate. The values are, however, within an order of magnitude of the observed field data which is the precision Billen (1976) claimed for the method.

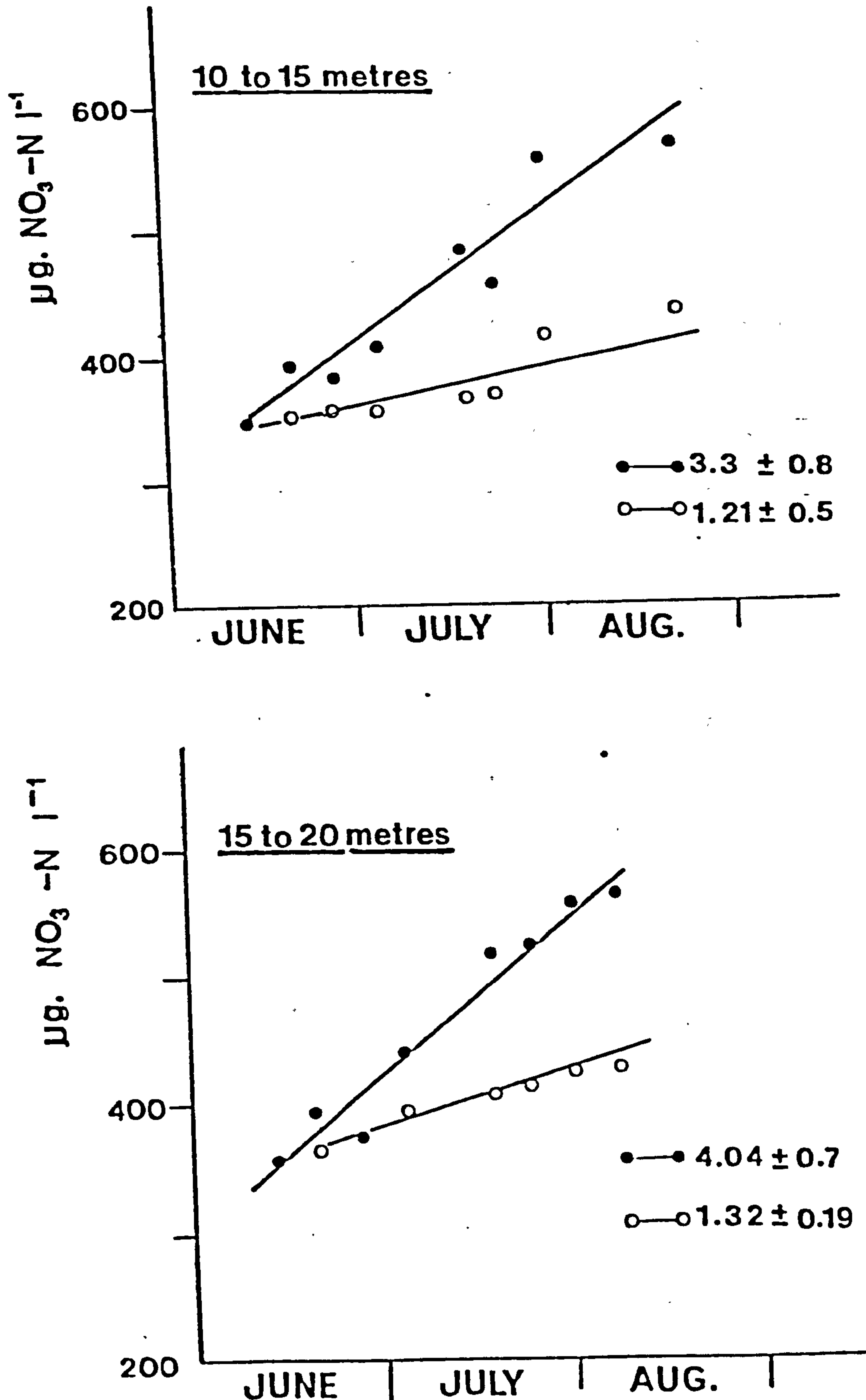
The field nitrate concentration data fluctuate to some degree between May and June, indicating that nitrification is not the dominant process at this time. These fluctuations affect the slope of the calculated regression line and therefore a more valid comparison should be made when nitrification is the dominant process i.e. during the nitrification phase itself. The regression line of the field nitrate concentrations, on time, was calculated, using only the data for the nitrification phase, between mid June and August. From the corresponding estimates of the nitrification rates the cumulative nitrate total was calculated as before using the intercept of the field nitrate data as the time zero value. These regression lines are shown in Figure 5.12 along with the slopes and corresponding 95% confidence limits. It is observed that again the estimated nitrate accumulation is significantly lower ( $P > 0.001$ ) than the actual field data. The divergence of the slopes is greater than when using the overall data and this is probably a consequence of the failure to detect high rates of nitrification throughout the whole nitrification phase.

## Discussion

Although efforts were made to mix the sample thoroughly prior to sub-sampling into the 135 ml bottles the results still showed considerable variation within treatments. Other workers who have used this method on water samples (Christofi, 1978; Somville, 1978) used single non-replicated N-Serve and ethanol treatments. If replicate treatments are not used some

Figure 5.12 Comparison of regression lines of field nitrate concentrations (●) and cumulative nitrate concentration (○) (calculated from estimates of the nitrification rate), both on time, in 10 - 15 m and 15 - 20 m depth layers of Grasmere 1977 during the nitrification phase.

The slope and its 95% confidence limits of each line are also shown. The intercept of the regression line for field nitrate concentrations was used as the initial nitrate concentration for the estimated cumulative total.



differences in CO<sub>2</sub> uptake will occur by chance and this natural variability, inherent in the data, will go undetected.

One possible source of error in the results arises from the use of hydrochloric acid to rinse, and remove uncombined bicarbonate from the cellulose ester membranes. This could result in loss, either through leaching or volatilization, of various labelled organic materials from the bacterial cells. An improvement on this technique has been described by Williams (1970), in which two membranes are placed on the filter holder. The lower membrane acts as a control since it is likely to retain as much soluble <sup>14</sup>C-bicarbonate as the upper membrane which also retains the particulate material. The separate counting of both membranes gives a simple count correction, although the number of samples to be counted is doubled. This method will not correct for any chemical absorption of <sup>14</sup>C-bicarbonate to inert particulate material in the sample.

Recently, Hall and Murphy (1980) have criticised the N-Serve with <sup>14</sup>C-bicarbonate method for estimating nitrification rates because the N-Serve only inhibits phase I organisms and therefore phase II organisms are still active. However, these authors were unable to suggest what were the alternative sources of energy yielding substrate (nitrite) for the phase II organisms. Belser (1977) and Miyazaki et al (1977) have noted nitrite production during nitrate reduction, in soil and marine environments respectively. Horsley (1978) found aerobic heterotrophic bacteria, capable of producing nitrite from nitrate, in culture at least, to be a predominant part of the viable aerobic heterotrophic population in Grasmere water samples. It is not known if these populations carry out this reaction in situ.

Although the present results are within an order of magnitude of the observed nitrate data I feel it would be worthwhile discussing the conditions under which the method may underestimate the actual nitrifying activity.

1. It is possible that both nitrifying or denitrifying heterotrophic bacteria are actively producing nitrite in situ and therefore allowing phase II organisms to be active in the presence of N-Serve, and thus assimilating CO<sub>2</sub>. Castignetti and Gunner (1980) demonstrated that a heterotrophic nitrifying bacterium, producing nitrite from pyruvic oxime, can initiate a synergistic relationship with Nitrobacter agilis, to produce nitrate, under culture conditions. However, from published carbon fixed to nitrogen oxidised values (see later) for both phase I and II organisms, the contribution of the nitrite oxidising chemolithotrophs is only 17 to 20% of the total CO<sub>2</sub> uptake and therefore is not likely to account for the present results.

2. It is now well established that the methane oxidising bacteria are able to oxidise ammonia to nitrite (O'Neil and Wilkinson, 1977; Rudd and Taylor, 1980) but the extent of their contribution to nitrification in the aquatic environment is unknown (Rudd and Taylor, 1980). It is also known that these organisms are sensitive to inhibitors of the true nitrifying organisms (Hubley, 1975).

The methylotrophs can be classified on the basis of their carbon assimilation pathways (Whittenbury and Kelly, 1977). They require carbon dioxide as an essential carbon source in addition to the major reduced carbon sources, methane, formaldehyde or methanol (Whittenbury and Kelly, 1977; Fenchel and Blackburn, 1979; Brock, 1977). Brock (1977) also states that this carbon dioxide uptake is small in relation to the carbon dioxide uptake of autotrophic bacteria via the Calvin-Benson cycle. Therefore, if the methane oxidising organisms are actively oxidising ammonia to nitrite in situ the observed N-Serve sensitive CO<sub>2</sub> uptake would be less than for the true nitrifying bacteria. This would result in an underestimation of the true nitrification rate. Nitrification by the methylotrophs can only be significant in environments where ammonia is present at high concentrations

in relation to that of methane availability (Dalton, 1977) (e.g. if methane is completely absent and ammonia present in  $\mu\text{g}$  quantities). Such conditions do exist in the hypolimnion of Grasmere and therefore activity of the methylotrophic bacteria in ammonia oxidation could account for the present results. Brock (1977), however, states that the methane oxidising bacteria cannot grow autotrophically using ammonia as the sole energy source. Romanovskaya (1976) could only correlate nitrite production to the biomass of Methylococcus thermophilus if methane was bubbled through the culture. Therefore, under conditions in the hypolimnion of Grasmere, the reduced carbon sources also essential for growth of methane oxidising bacteria, would not be present in sufficient concentrations to support maximum growth rates. Under these conditions  $\text{CO}_2$  uptake would be considerably less than expected.

3. There may be errors in the use of carbon dioxide uptake as an index of nitrogen oxidation by the chemolithotrophic nitrifiers. Aleem (1977) stated that the endergonic, carbon dioxide based, biosynthesis in chemolitho-autotrophs is tightly coupled to the exergonic inorganic oxidation reactions. Hamilton (1979) noted that when  $\text{CO}_2$  was absent lithotrophic oxidation, by hydrogen bacteria, was considerably reduced. On the other hand, Elsdon (1964) commented that energy metabolism and  $\text{CO}_2$  fixation can be separated in time due to the accumulation of storage products, which can be metabolised. Tobbach and Laudelot (1965) have shown polyhydroxy butyrate to fulfill this role in Nitrobacter.

It is also well established that both Nitrosomonas and Nitrobacter utilize organic compounds in the presence of their specific substrates (Wallace et al, 1970). Complete heterotrophic growth has been reported for Nitrobacter isolates (Smith and Hoare, 1968; Josserand and Cleyett-Marrel, 1979; Kaltoff et al, 1979; Bock, 1976; Steinmuller and Bock, 1977) although growth rates have been considerably less than under

lithotrophic conditions. Under mixotrophic growth conditions (organic and inorganic substrates present) Garretson and San Clemente (1978) noted increased yields of N. agilis over those obtained in autotrophic culture when only CO<sub>2</sub> is present. In the presence of acetate CO<sub>2</sub> assimilation decreased. Steinmuller and Bock (1977) noted that pyruvate (in the presence of nitrite) decreased the activity of ribulose biphosphate carboxylase by 50% in N. agilis (this enzyme has also been demonstrated to be the main CO<sub>2</sub> assimilating enzyme in Nitrosomonas, Roa and Nicholas, 1966). Tricarboxylic acid cycle enzymes had essentially the same activities under autotrophic and mixotrophic growth conditions. The natural environment is a mixotrophic growth environment and when organic carbon monomers are available it is the more efficient (energetically) for the organisms to assimilate these rather than fix CO<sub>2</sub>. Assimilation represses CO<sub>2</sub> uptake resulting in an underestimation of nitrifying activity by the <sup>14</sup>C-bicarbonate and N-Serve method.

4. McLaren (1971) divided the conserved energy budget of the cell into growth and maintenance requirements. Recently Belser (1979) pointed out that with a constant supply of ammonia, a nitrifying population would only increase in numbers until the substrate supply was sufficient to meet the maintenance energy requirements of that population. Under such conditions the carbon dioxide uptake could be less than under conditions of active growth.

5. The calculated N-Serve sensitive carbon uptake may be transformed to a value for nitrogen oxidised using a conversion factor. Such transformations, reported in the literature (Billen, 1976; Somville, 1978; Christofi, 1978) used factors which varied between 9 and 10. This factor is either the ratio of measured carbon fixed to nitrogen oxidised in fully nitrifying cultures (ammonia oxidised to nitrate



without nitrite accumulating) or the sum of the C:N ratios of both phase I and II cultures. A list of published C:N ratios for both phase I and II organisms is shown in Table 5.4 (where a range of values was reported the mean value is presented). From these data the calculated mean C:N ratios for phase I and phase II cultures have standard deviations of 73% and 70% respectively. This suggests that any one of a wide range of values could, justifiably be used. Figure 5.13 shows the good agreement with the field data from Grasmere if a conversion factor of 15 is used to transform the calculated N-Serve sensitive carbon uptake to nitrogen oxidised. The C:N ratio of an enrichment, or culture, of a nitrifying bacterium is a measure of the growth yield i.e. the amount of cell substance formed during the oxidation of unit concentration of substrate. Under standard conditions this growth yield, also known as the yield coefficient, is constant for a particular organism. Some of the variability noted in Table 5.5 can be accounted for by the differences in conditions under which the C:N ratios were measured. It would not serve a useful purpose to review all the culture conditions used but variables such as temperature, medium composition or incubation time (as one substrate may become limiting) may determine the magnitude of measured C:N ratios. The growth yield will also depend on the biomass of the organisms present. Recently, Belser and Schmidt, (1980) have noted differences in growth kinetics, which could be related to different cell biomass, not only between genera of phase I organisms but also within the genus Nitrosomonas. Therefore, however straightforward the conversion factor may seem, it is apparent that values obtained from cultures cannot be related directly to field situations. The most satisfactory solution is to measure this ratio directly on samples taken during the investigation but this is not always possible because of low activities and the imprecision of chemical

Table 5.5. Published carbon fixed to nitrogen oxidised values (in moles) for phase I and II lithotrophic nitrifying organisms. The mean of these values and its standard deviation are also presented.

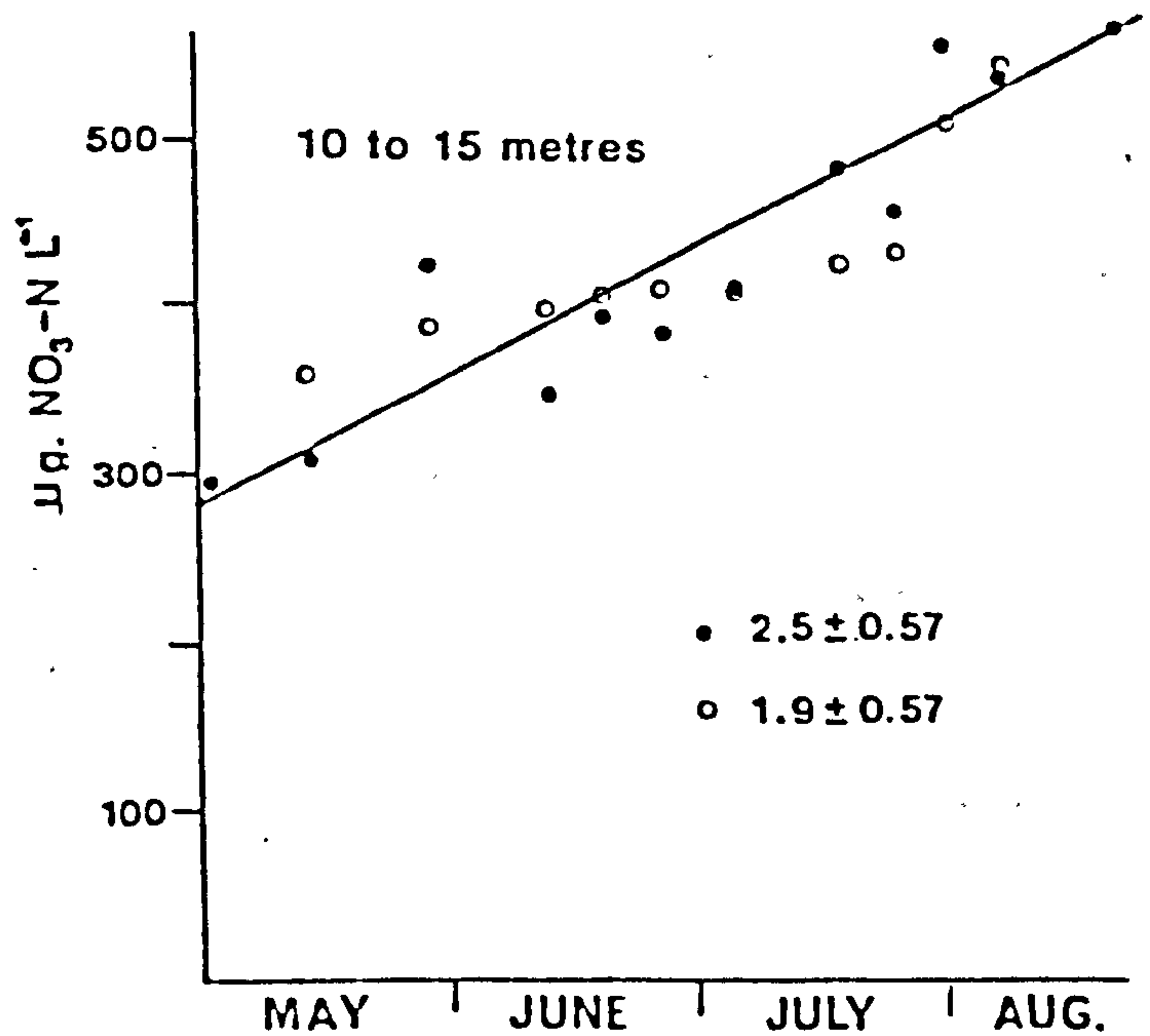
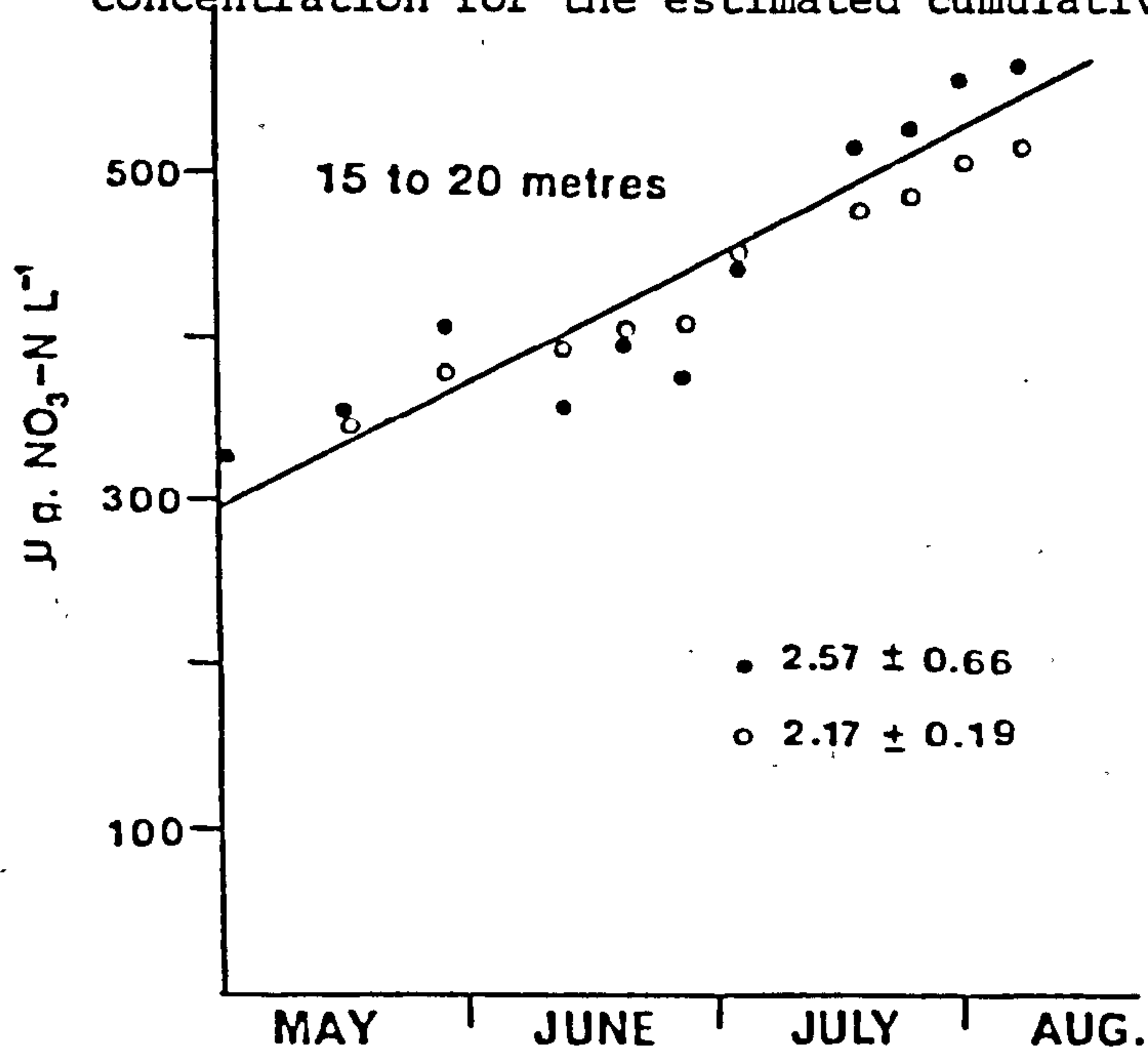
Reference	Phase I <sup>(a)</sup>	Phase II <sup>(a)</sup>
Lees (1955)	0.1445	
Winogradsky (1890)	0.0334	
Hofman and Lees (1952)	0.1804	
Aleem and Alexander (1960)		0.0022
Loveless and Painer (1968)	0.0448	
Carlucci and Strickland (1968)	0.0796	
Painter (1970/77)	0.1109	0.0233
Meyeroff (1918)	0.010	
Wezernak and Gannon (1968)	0.0575	0.0098
Laudelot <u>et al</u> (1968)	0.0386	0.0164
mean	$\bar{x}$ 0.0777	$\bar{x}$ 0.0129
standard deviation	s 0.0567	s 0.0090

(a) where a range of values were presented the mean value is reported.

Figure 5.13 Comparison of field nitrate concentrations (●) and cumulative nitrate concentration (○) (calculated from estimated nitrification rates) in the 10 - 15 m and 15 - 20 m depth layers of Grasmere. A factor of 15 was used to convert N-Serve sensitive CO<sub>2</sub> uptake to nitrogen oxidised.

Slopes of the regression lines and corresponding 95% confidence limits are shown.

The intercept of the regression line for field nitrate concentrations, on time, was used as the initial nitrate concentration for the estimated cumulative total.



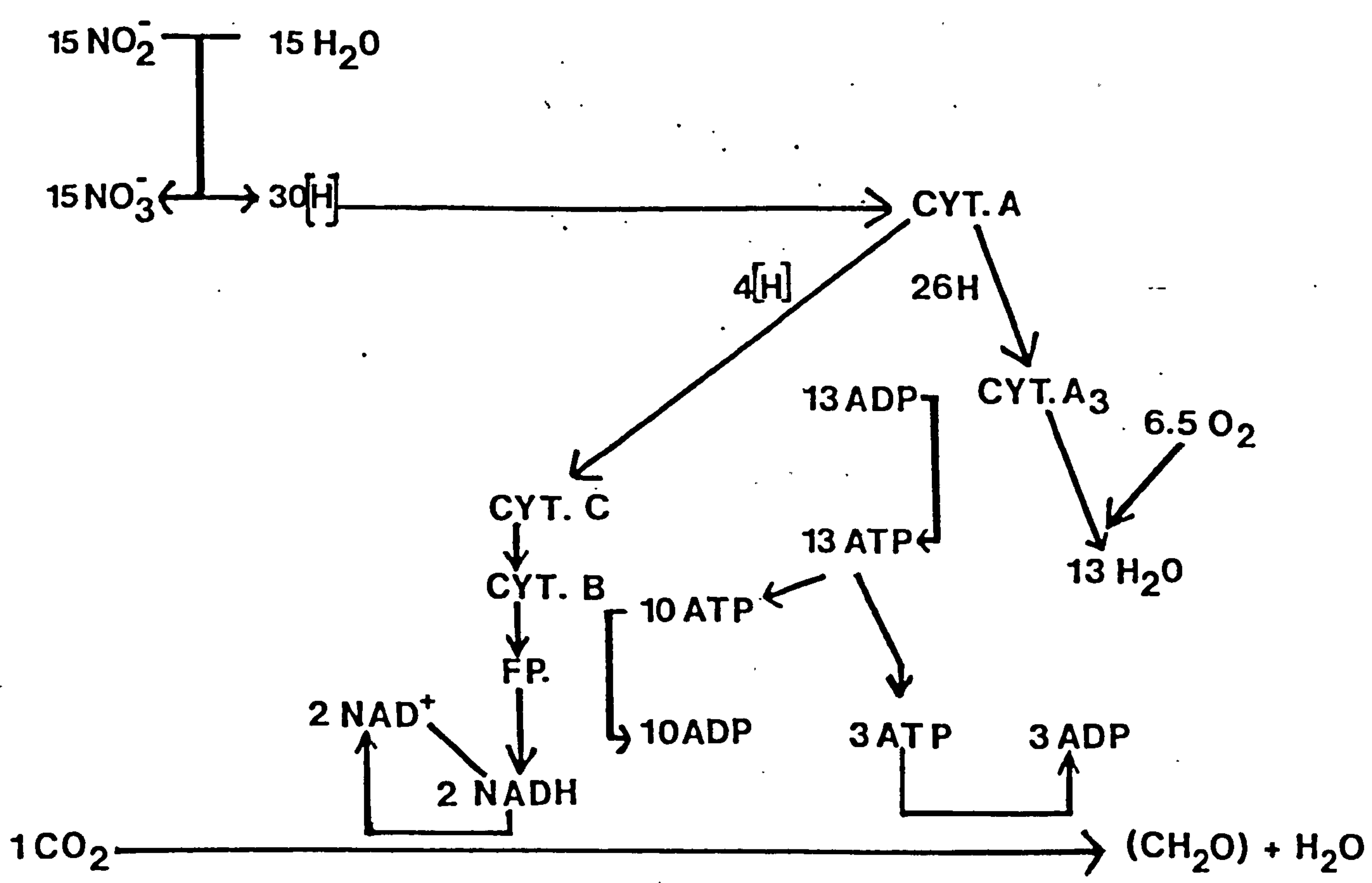
determinations. Christofi (1978) estimated the growth yield of a nitrifier enrichment but it is not known whether conditions of enrichment select organisms which are dominant in the field. It would seem useful therefore to discuss to what extent the C:N ratio can be rationalized. Kelly (1978) presents a hypothetical scheme for the amount of nitrite which must be oxidised to nitrate by Nitrobacter to reduce one mole of CO<sub>2</sub> to the level of carbohydrate via the Calvin Benson cycle. This scheme assumes 1 mole of A.T.P. is produced per mole nitrogen oxidised, which appears to be the case (Aleem, 1977; Kiesow et al, 1972). The scheme is reproduced in Figure 5.14. When corrections are made for energy requiring growth related reactions a C:N ratio of 1:21 is obtained, which is approximately four to five times smaller than the figures presented in Table 5.4. The energy budget of the cell is not, however, confined to growth, and other components referred to as maintenance and 'energy leakage' i.e. that energy which is not conserved by the cell, are involved. The overall thermodynamic efficiency of growth, particularly of chemolithotrophic organisms, is generally rationalized as the 'free energy efficiency' defined in the equation of Baas-Becking and Parks (1927) as:

$$\frac{\text{Carbon assimilated} \times \text{standard free energy required}}{\text{Substrate oxidised} \times \text{standard free energy released}} \times 100$$

In other words, for the nitrifying bacteria, it is the product, expressed as an efficiency, of the measured C:N ratio and the ratio of the standard free energy changes associated with fixing the carbon and oxidising the nitrogen. However, this equation incorporates the portion of total energy available which is used for growth and not the portion of energy conserved by the cell which is used for growth. If the efficiency of free energy utilization was known it would be possible to substitute this energy value into the denominator of the above

Figure 5.14. Schematic representation for the minimum nitrite oxidation, by phase II lithotrophic nitrifying organisms, required to fix 1 mole of CO<sub>2</sub> to (CH<sub>2</sub>O) via the Calvin-Benson cycle (from Kelly 1978).

Note [H] = H<sup>+</sup> + e<sup>-</sup>



equation and therefore obtain a direct estimate of the energy partition between growth and other processes within the cell. For example, in the case of Nitrobacter, 1 mole of A T P is produced per mole N oxidised, therefore the standard free energy change ( $\Delta G^{\circ}$ ) for A T P hydrolysis should be substituted for the standard free energy release during substrate oxidation in the above equation. Then, knowing the fraction of conserved energy which is used for growth, and the minimum theoretical C:N ratio to fix 1 mole of  $\text{CO}_2$ , it should be possible to calculate a minimum total C:N ratio. However, this approach is subject to many criticisms. Firstly, although the measurements of the efficiency of free energy utilization (see Laudelot et al, 1968) are constant for a given cell suspension, there is considerable variation between cell suspensions (Laudelot pers. comm; Hooper pers. comm). Secondly, the calculation assumes that nitrogen oxidation is the only source of A T P within the cell. Finally, the use of standard free energy changes in such calculations cannot be related to in situ conditions. The sign and magnitude of  $\Delta G^{\circ}$  only gives information on the direction, and extent, to which a reaction will occur and to use these values to determine energy partitioning is not valid. For these reasons no further rationalization of the C:N conversion factor was attempted in this thesis.

#### 5.4 Interlake comparisons

Because of the significant contribution of nitrification to the oxygen deficit of the hypolimnion of Grasmere a study was undertaken to investigate the frequency, and quantitative significance, of nitrification in the hypolimnia of lakes in this area. Four lakes were chosen for this study, the fifth, as control, was Grasmere. The geological and hydrological characteristics of these lakes have been described earlier (Section 4.1) but it would be worthwhile to examine the reasons behind their choice.

Pearsall (1921) first considered the lakes of the English Lake District as a series and arranged them in order of increasing eutrophication. This classification was based on the percentage of drainage system which was cultivated, the amount of lake shore which was not silted and the maximum depth of penetration of light. Since that time it has been recognized that the morphometry of the lake, in addition to the surrounding geology and land use, is also important in determining its trophic status. Many studies have now been carried out on this series of lakes and it has been found that a number of biological (Jones, 1972; Jones et al; Gorham et al, 1974), chemical (Jones, 1972; Mackereth, 1957) and physical (Talling, 1971; Jones et al. 1979) parameters can be correlated with the trophic status of the lakes. The agreement is good at the extreme oligotrophic and eutrophic ends of the scale and only minor differences in the ranking of the middle order were found. The series of 16 lakes in increasing order of eutrophication and their maximum and mean depths, are shown in Table 5.6. Only Esthwaite and Blelham Tarn are more eutrophic than Grasmere and these are the most studied lakes in this area. As discussed in the literature review, nitrification has been demonstrated in Blelham Tarn (Jones and Simon, 1980; Cristofi, 1978) and data were available from earlier studies on Esthwaite water. Therefore little would have been gained by including these lakes in the present study. Paerl (1975) observed nitrification in oligotrophic Lake Tahoe and so lakes of low trophic status in the present series were considered worthy of study. Buttermere and Derwentwater were chosen because of their morphometric similarities to Grasmere, particularly with regard to maximum depth. Windermere (North basin) was chosen to represent a deep lake. Brotherswater is a lake not included in the Pearsall series but has many characteristics similar to Grasmere. It is relatively small, situated on similar geological formations and has a large volume of epilimnion in relation to that of the hypolimnion (like Derwentwater also).

Table 5.6 The Pearsall Series of lakes in the English Lake District,  
arranged in order of increasing eutrophication.

	<u>Lake</u>	<u>Maximum depth (m)</u>	<u>Mean depth (m)</u>
1.	Wastwater	76	39.7
2.	Ennerdale	42	17.8
3.	Buttermere	28.5	16.6
4.	Crummock Water	44	26.7
5.	Coniston	56	24.1
6.	Thirlmere	46	16.1
7.	Windermere (N)	64	25.1
8.	Ullswater	62.5	25.3
9.	Derwentwater	22	5.5
10.	Bassenthwaite	19	5.3
11.	Windermere (S)	42	16.8
12.	Loweswater	16	8.4
13.	Rydal Water	19	
14.	Grasmere	21.5	7.7
15.	Esthwaite	15.5	6.4
16.	Blelham Tarn	14.5	6.8



Samples were taken 1 m and 5 m above the sediments, at the deepest point of the lakes, at monthly intervals, and analysed for ammonia and nitrate concentrations with 6 replicate determinations per sample. The nitrification rate was determined as previously described but this time using six replicate bottles per treatment and an incubation time of only 3 h. The shorter incubation time was used in response to the report by Somville (1978) who found nitrification could be detected, by this method, in water samples from the Scheldt estuary, using incubation times of 3 to 6 h.

None of the lakes, except Grasmere and Windermere had marker buoys to indicate the deepest point. In order to find this site a distance rangefinder and prominent land bearings were used. Using this method a conservative estimate of the accuracy of position finding would be within a horizontal distance of  $\pm 20$  m. Because of this variability each lake was sampled to estimate the spatial variation of inorganic nitrogen concentrations at least once in the sampling period. Three sites, on each lake, were sampled, both 1 m and 5 m above the sediments, within 10 to 25 m (horizontal distance) of the estimated sampling position. Ammonia and nitrate determinations on these samples were analysed for variation without data transformation. The results are shown in Table 5.7. Ammonia was undetectable in Buttermere and only nitrate values are presented. Analysis of variance indicated no significant differences ( $P > 0.05$ ) between the three sites on each lake. It was not considered necessary to make such observations on Grasmere or Windermere although wind direction could affect the position of the sampling boat. Changes in concentrations greater than the confidence limits for each analysis ( $P = 0.05$ ) can be regarded as real temporal variations.

The results obtained, from each lake, with samples taken 1 m and 5 m above the sediments are presented in Figures 5.15 and 5.16 respectively.

Table 5.7 Spatial variation, on the dates specified, of Nitrate and Ammonia concentrations 1 m and 5 m above the sediments of Brotherswater, Buttermere and Derwentwater.

All concentrations in  $\mu\text{g N l}^{-1}$ .  $\bar{x}$  mean of 6 determinations per sample

s standard deviation of  $\bar{x}$

$\bar{X}$  the overall mean

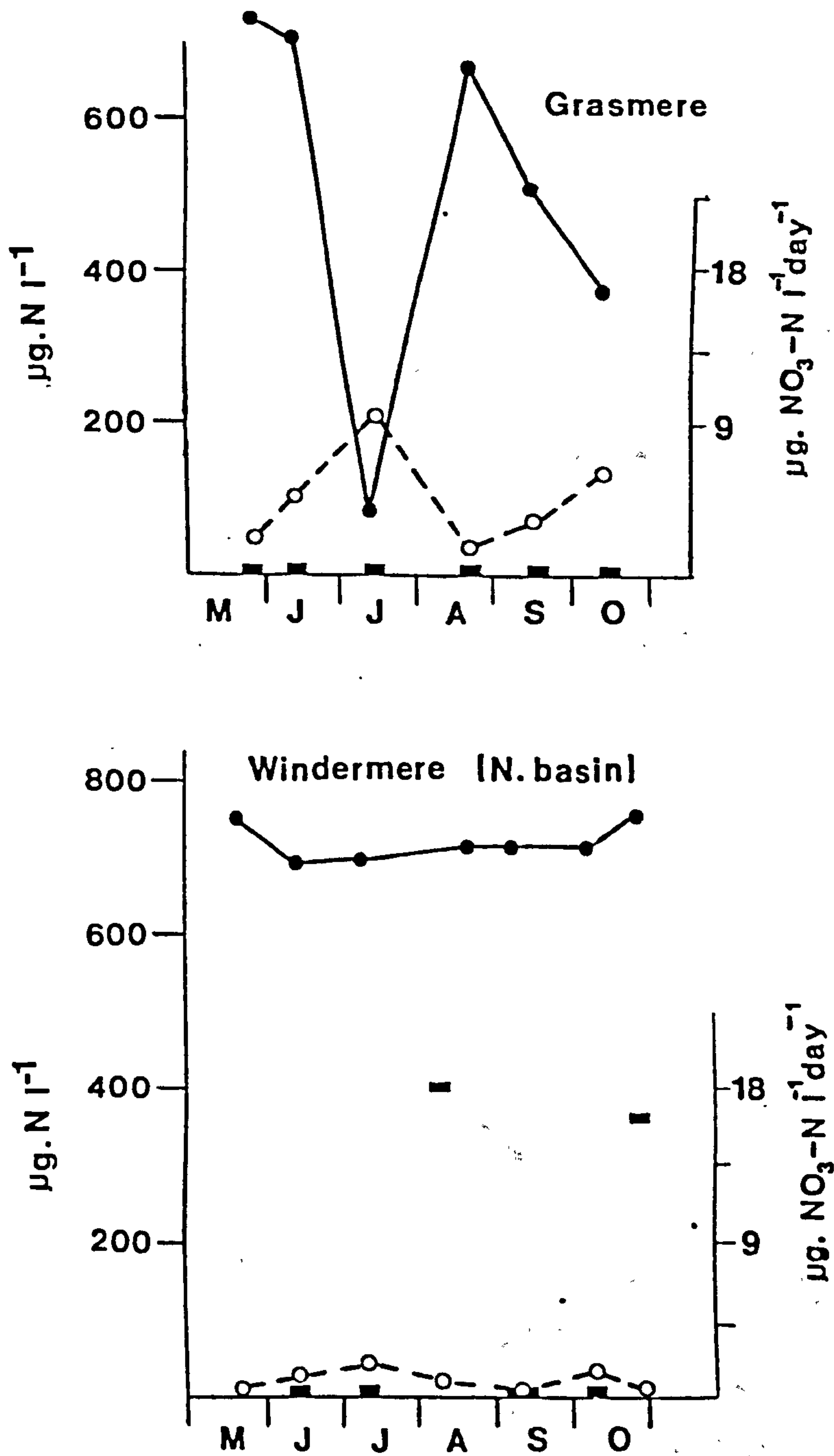
S standard deviation of  $\bar{X}$

		BROTHERSWATER (15.8.79)							
		SAMPLE 1		SAMPLE 2		SAMPLE 3		OVERALL	
		$\bar{x}$	S	$\bar{x}$	S	$\bar{x}$	S	$\bar{X}$	S
1m above sediment	NH <sub>3</sub> -N	388	5	402	17	398	5	397	13
	NO <sub>3</sub> -N	95	7	88	3	103	6	96	8
5m above sediment	NH <sub>3</sub> -N	182	1	185	1	189	3	182	3
	NO <sub>3</sub> -N	254	12	273	14	265	17	264	16

		BUTTERMERE (9.7.79)							
		SAMPLE 1		SAMPLE 2		SAMPLE 3		OVERALL	
		$\bar{x}$	S	$\bar{x}$	S	$\bar{x}$	S	$\bar{X}$	S
1m above	NO <sub>3</sub> -N	348	8	351	7	352	7	350	7
5m above	NO <sub>3</sub> -N	353	10	352	7	353	10	352	9

		DERWENTWATER (14.6.79)							
		SAMPLE 1		SAMPLE 2		SAMPLE 3		OVERALL	
		$\bar{x}$	S	$\bar{x}$	S	$\bar{x}$	S	$\bar{X}$	S
1m above sediment	NH <sub>3</sub> -N	26	2	22	3	25	3	23	6
	NO <sub>3</sub> -N	418	32	416	15	420	26	418	24
5m above sediments	NH <sub>3</sub> -N	15	1	12	3	15	2	14	2
	NO <sub>3</sub> -N	429	36	425	16	424	18	426	23

Fig. 5.15 Monthly variation of ammonia (o---o) and nitrate (●—●) concentrations and the rate of nitrification (■, as  $\mu\text{g NO}_3\text{-N produced l}^{-1} \text{ day}^{-1}$ ), 1 m above the sediments in a number of lakes.



Samples taken at the deepest point in each lake.

Fig. 5.15 continued

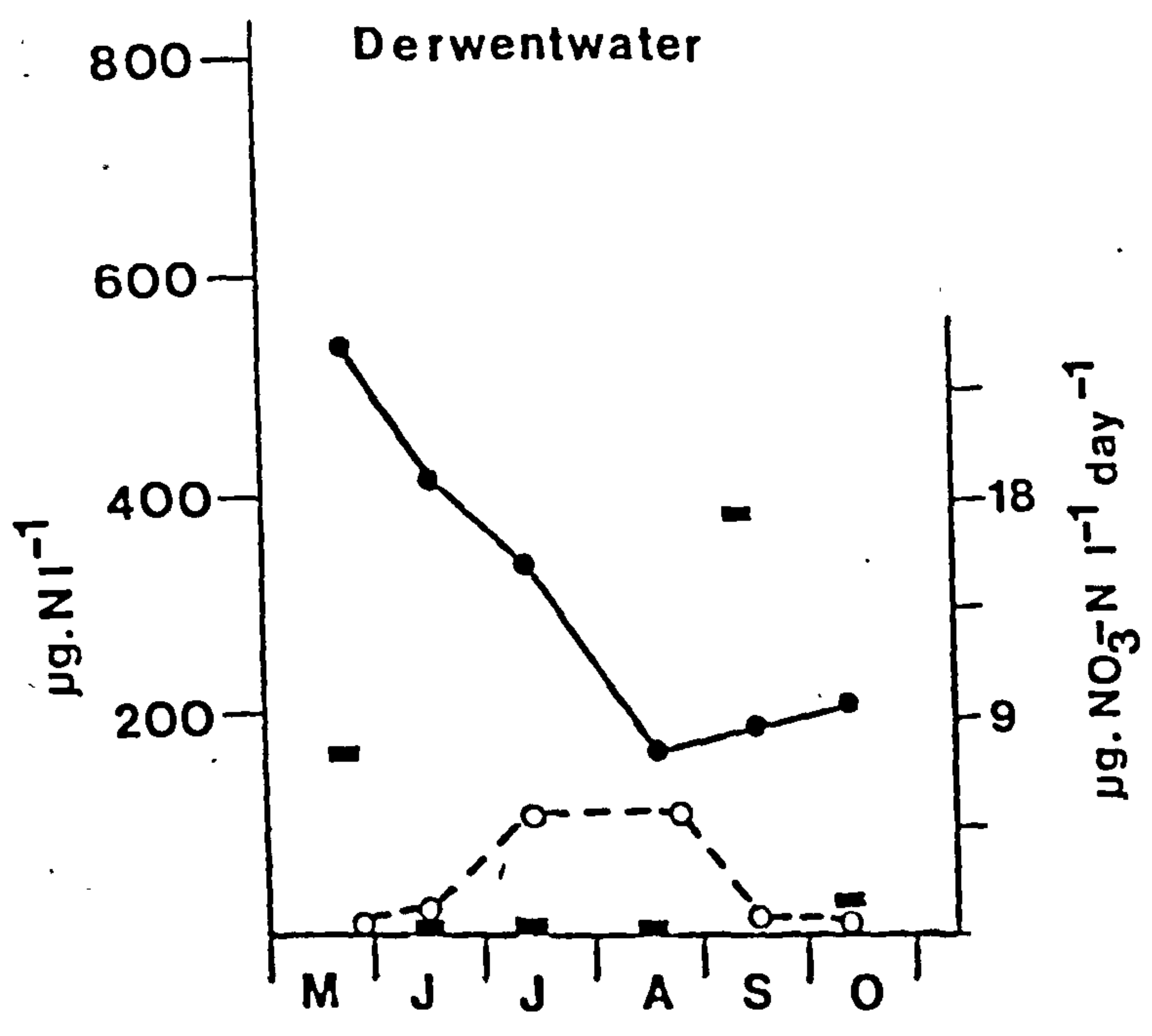
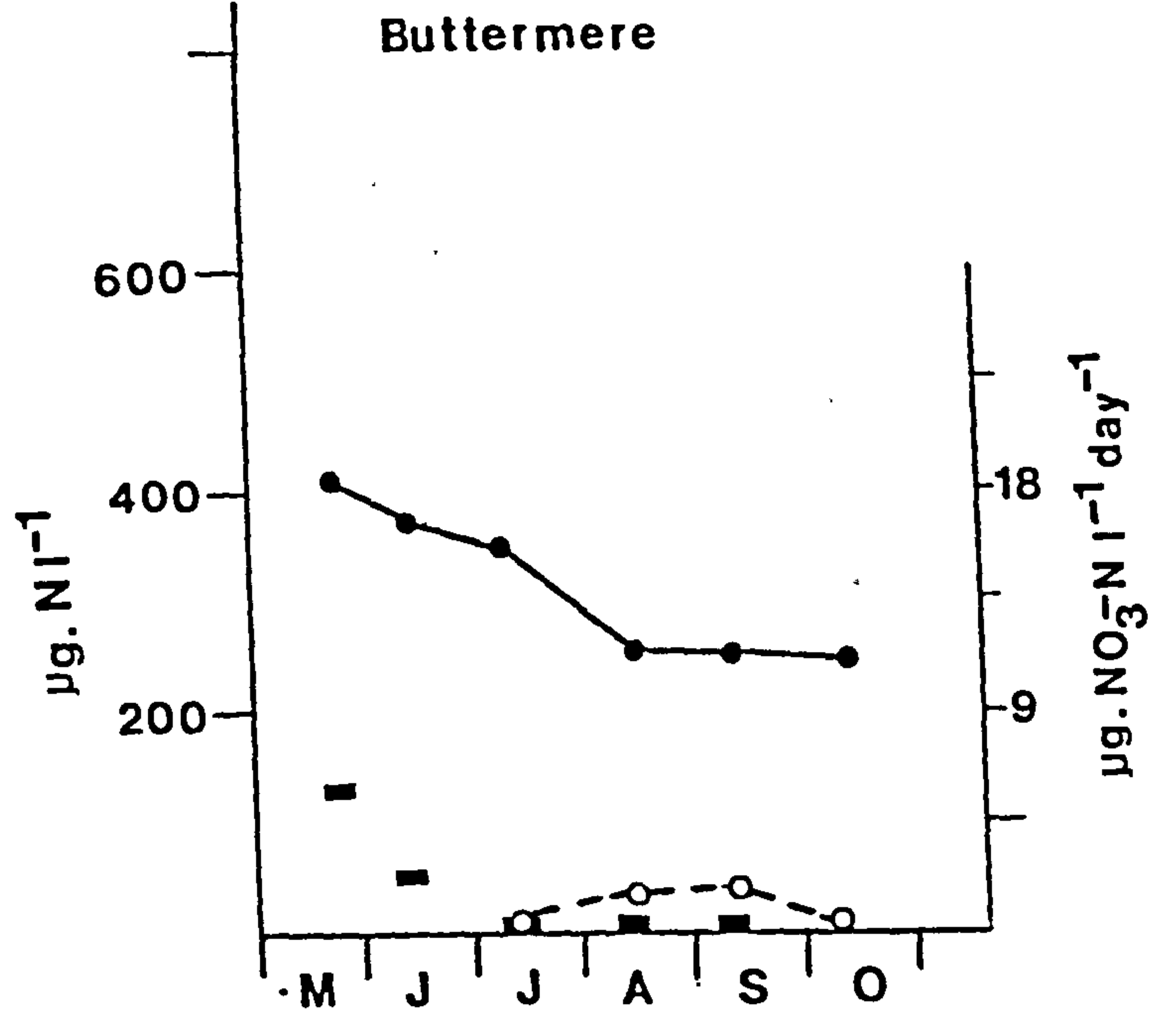
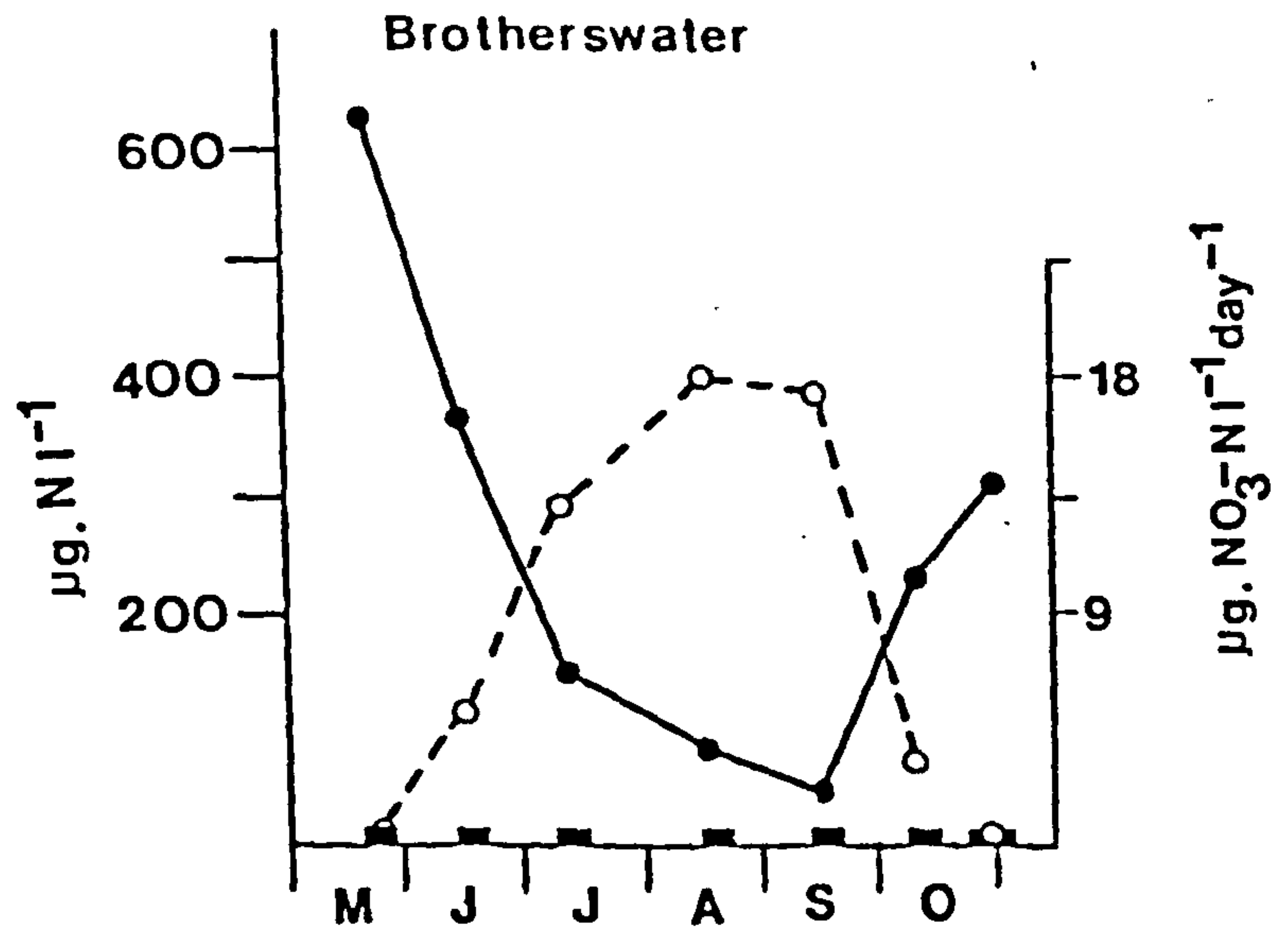


Fig. 5.16 Monthly variation of ammonia (o---o) and nitrate (●—●) concentrations and the rate of nitrification (■, as  $\mu\text{g NO}_3\text{-N l}^{-1} \text{ day}^{-1}$  produced) 5 m above the sediments in a number of lakes. Samples taken at the deepest point in each lake.

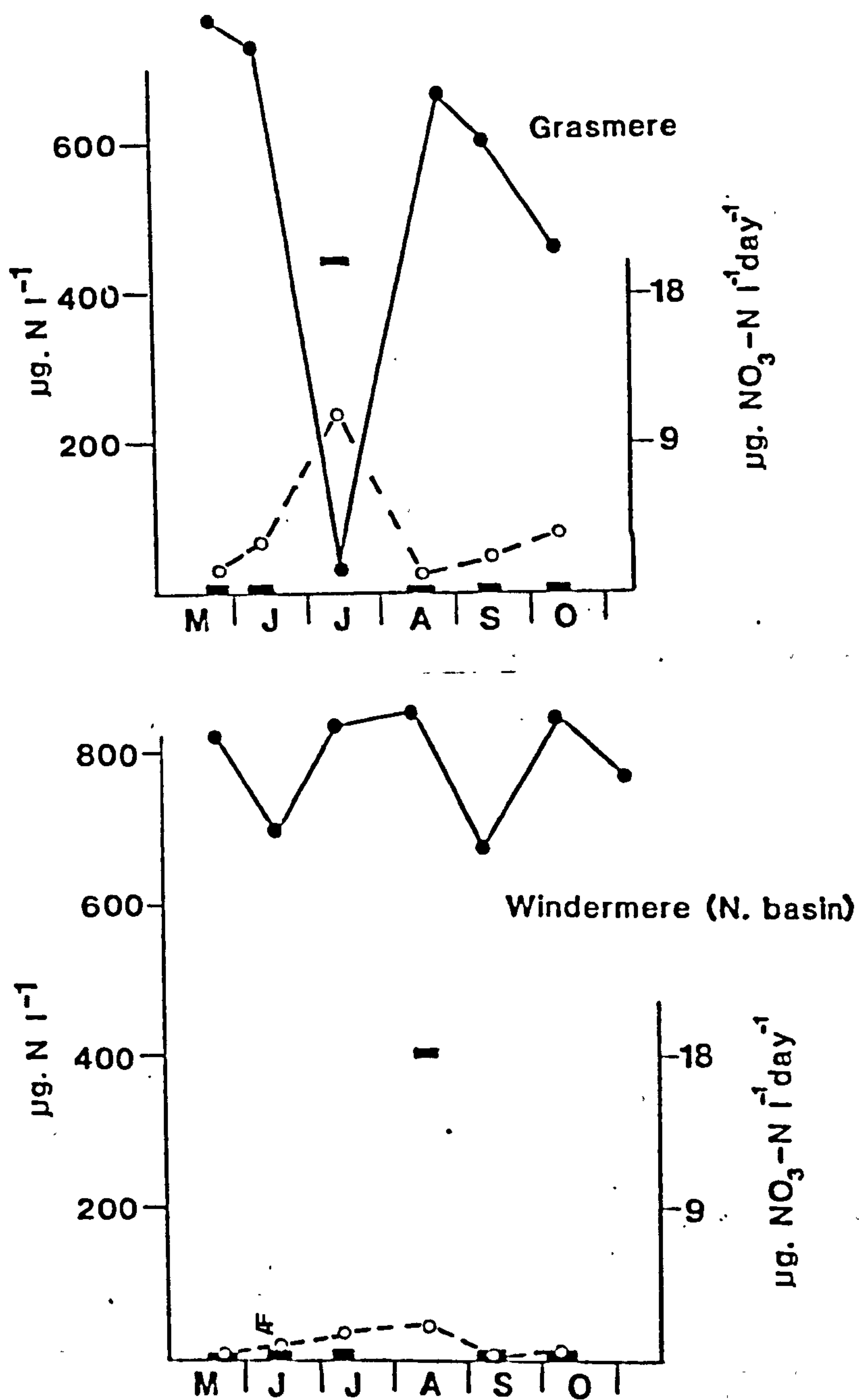
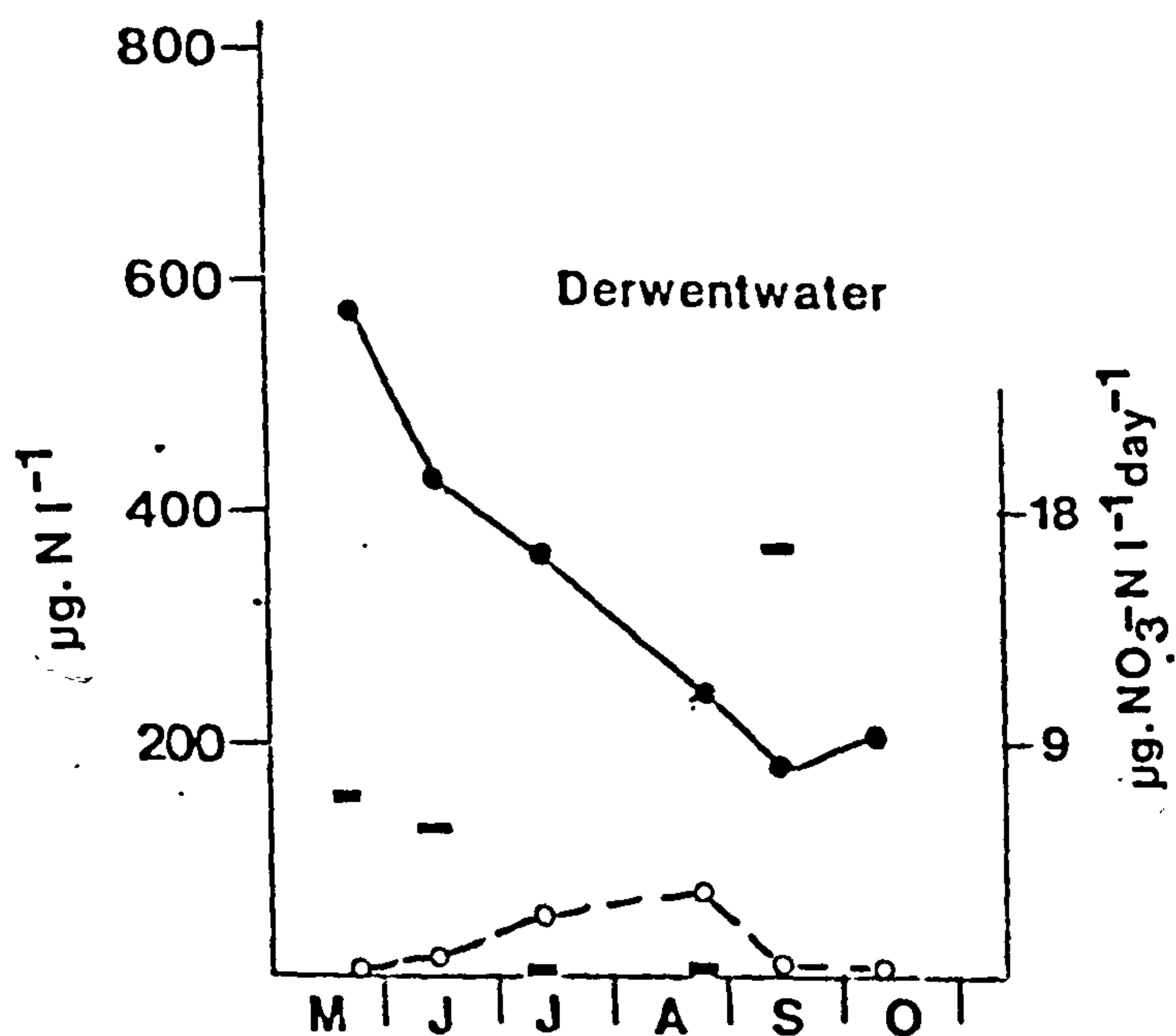
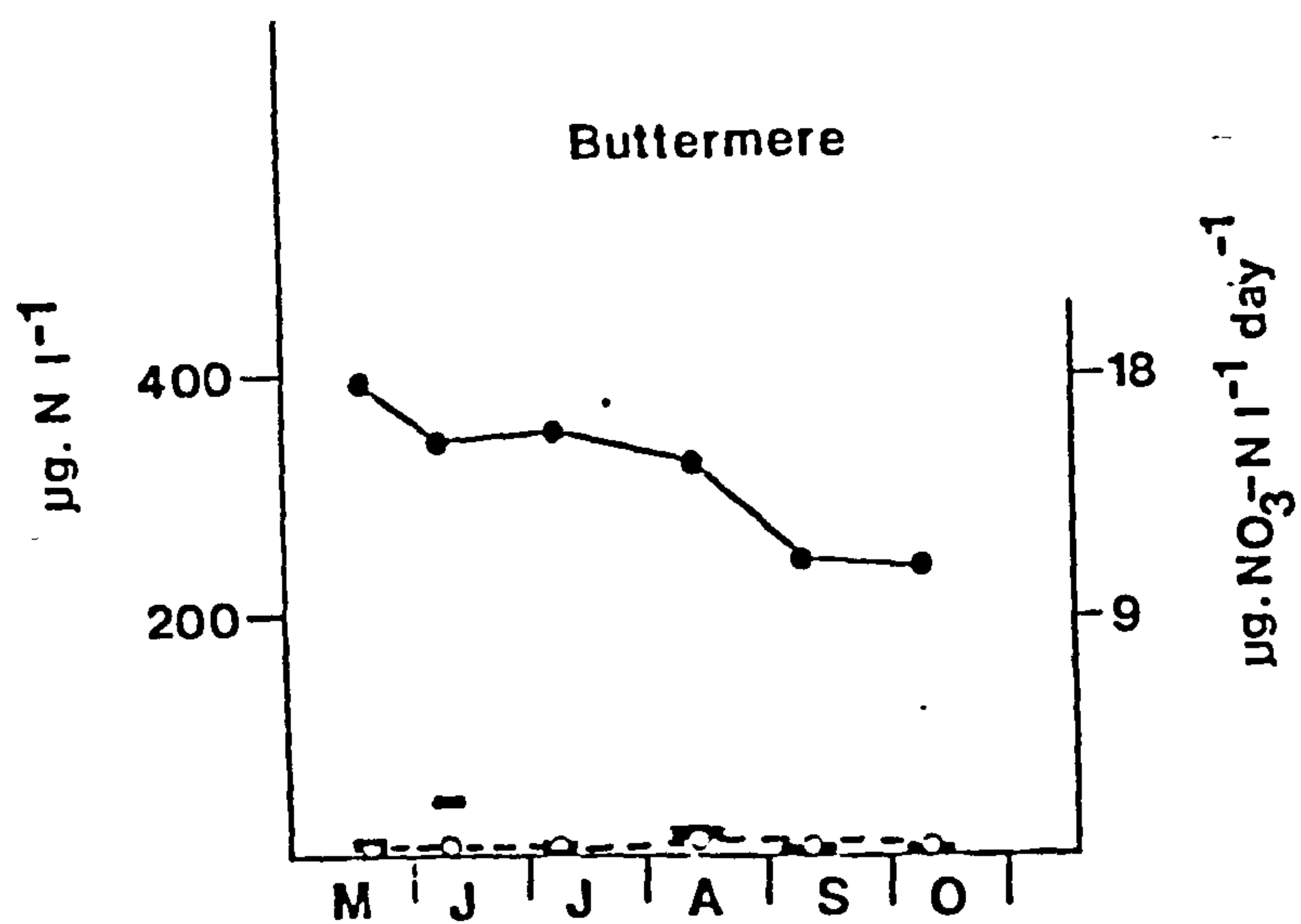
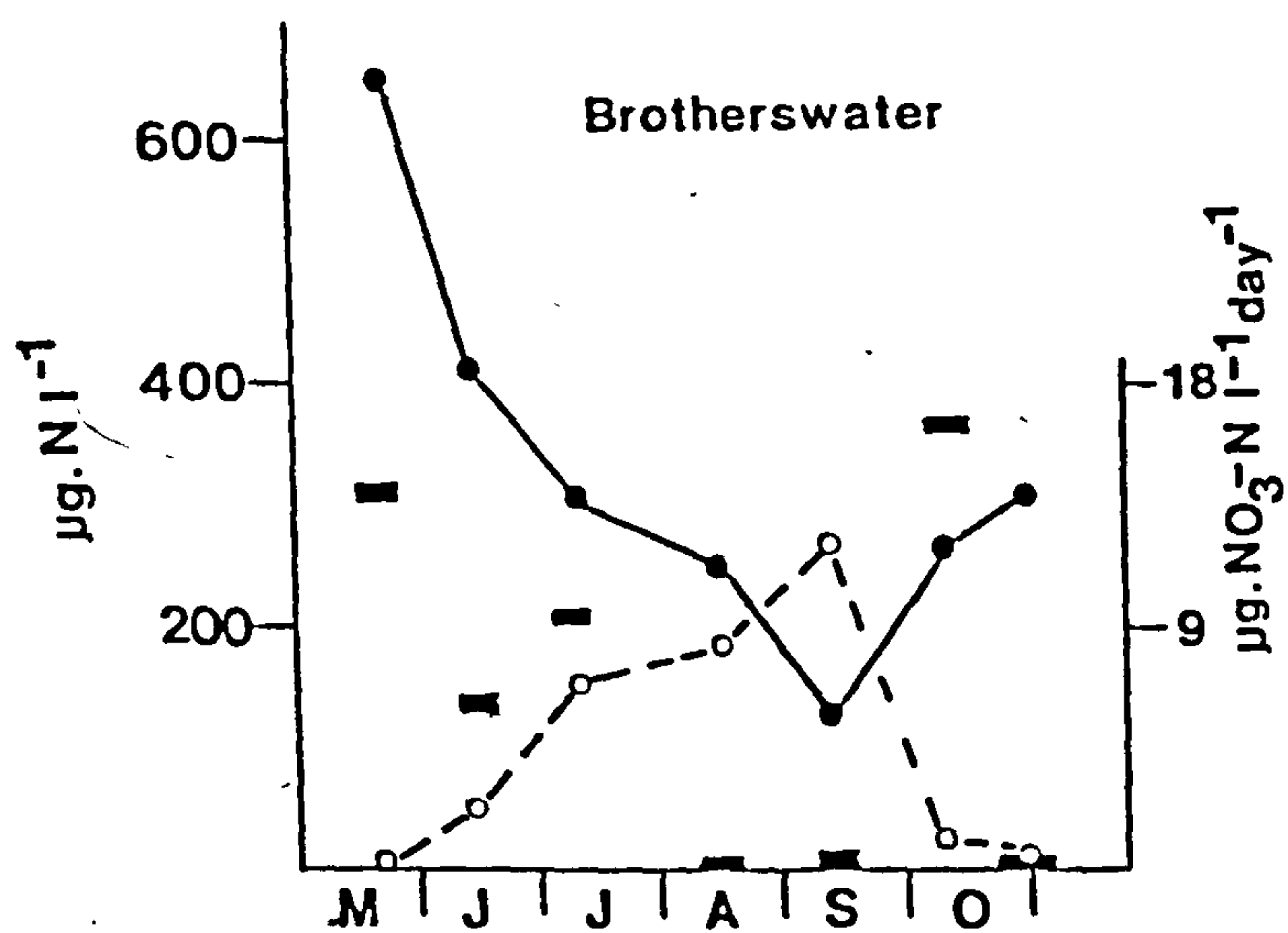


Fig. 5.16 continued



#### 5.4.1 Inorganic nitrogen

In all the lakes, with the exceptions of Brotherswater and Grasmere, the nitrate concentration always exceeded that of ammonia both 1 m and 5 m above the sediments. In Buttermere and Windermere ammonia was either absent, or present in low concentrations ( $38 \mu\text{g N l}^{-1}$  maximum). All lakes showed the tendency for more ammonia to accumulate closer to the sediment water interface. The highest ammonia concentration ( $401 \mu\text{g N l}^{-1}$ ) was recorded in Brotherswater in August, whilst the maximum nitrate concentration ( $750 \mu\text{g N l}^{-1}$ ) was consistently found in Windermere. The lakes in which ammonia was detected in significant concentrations (i.e.  $> 50 \mu\text{g N l}^{-1}$ ) all showed periods of apparent nitrification, with decreasing ammonia concentrations concomitant with increasing nitrate concentrations at some time during the season. Grasmere showed the typical pattern of previous years which has already been discussed. Nitrification apparently occurred in Brotherswater between September and October whilst the results from Derwentwater indicated the period of nitrification between August and September. The only lake to show different patterns of nitrate concentrations 1 m and 5 m above the sediments was Windermere, where concentrations were constant at the deeper sampling site, but at the 5 m site considerable variation was noted. Possibly two periods of nitrate production could be observed, at this latter site, but neither were reflected in ammonia concentrations. All lakes, with the exception of Windermere, showed decreasing nitrate concentrations throughout the season, or until the nitrification phase. This observation, in conjunction with the low ammonia concentrations, possibly suggests that in Windermere nitrification was occurring throughout the summer months but never became the dominant process.



#### 5.4.2 Nitrification rates

Differences were noted between the two sampling sites in each lake and therefore these will be discussed separately.

##### 1 m above sediments

Although the ammonia and nitrate concentrations in both Brotherswater and Grasmere showed typical nitrification patterns, nitrification rates remained undetectable throughout the sampling period. This was particularly surprising for Grasmere as the previous observations suggested peaks of nitrifying activity in May and July. The other three lakes all showed detectable nitrification rates at sometime throughout the period of stratification, usually associated with changes of inorganic nitrogen concentrations typical of nitrification, but not in all cases. Derwentwater showed peaks of activity in both May and September, only the latter being associated with both increasing nitrate, and decreasing ammonia, concentrations. Nitrifying activity in Buttermere decreased from May to June and the complete loss of activity in July was associated with the appearance of detectable ammonia concentrations and an increased rate of nitrate depletion in the hypolimnion. In Windermere there was no early season activity but peaks were noted in August and October, again only the latter being associated with increasing nitrate, and decreasing ammonia concentrations.

##### 5 m above sediments

In contrast to the 1 m data nitrification rates were detected in all the lakes at sometime during the season. Derwentwater again showed both early and late season (September) peaks of activity, but, in contrast to the deeper samples, the early season activity was extended into June. Chemical changes, indicative of nitrification, were only associated with the late season activity. Nitrifying activity in Buttermere was restricted to early in the season and this correlated

with a decrease in the rate of nitrate removal from the hypolimnion. Windermere was restricted to activity only in August and this showed no relation to associated changes of inorganic nitrogen concentrations. Grasmere displayed a peak of nitrifying activity in July, also noted in the more detailed survey of this lake in 1977. This activity coincided with the minimum recorded nitrate concentration at this depth. Although apparently contradictory, this emphasised the dangers inherent in using concentrations of inorganic nitrogen species as indicators of the nitrogen cycle processes occurring. The increase in nitrate concentration which was detected over the next month demonstrated that nitrification had occurred. The results also show the inaccuracies associated with the use of such a low sampling frequency. Brotherswater showed both early and late season peaks, the latter coinciding with the apparent nitrification phase noted from changes in inorganic nitrogen concentrations.

#### Discussion

It was interesting to note that those lakes in which substantial concentrations of ammonia accumulated in the hypolimnion developed patterns of inorganic nitrogen concentrations typical of nitrification. As the water columns contained oxygen during the accumulation of ammonia the implication is that ammonification of organic nitrogen is the source as fermentative processes could not be active. This pattern of increasing ammonia concentrations, followed by typical nitrification chemical changes, was noted during stratification of Grasmere in 1975 to 1977 and also in Blelham Tarn (Christofi, 1978; Jones and Simon, 1980). The available data for Esthwaite (Heaney, unpublished data), the most eutrophic lake in this area, shows that during the summer of 1972 ammonia concentrations increased at all depths below 6 m from mid June when sampling commenced. This accumulation continued, and nitrate concentrations declined, throughout July at depths of 12 m and below as

oxygen became depleted. However, at the intermediate depths of 8 and 10 m ammonia concentrations declined from 120 to  $2 \mu\text{g N l}^{-1}$  concomittant with nitrate concentration increases from 80 to  $100 \mu\text{g N l}^{-1}$ , between July 17th and 31st. As oxygen was still present at these depths nitrifying activity is indicated. In the surface waters (0 - 6 m) both ammonia and nitrate concentrations declined throughout the period of stratification. All these lakes, which showed nitrification patterns have the characteristic of a small volume of hypolimnion relative to that of the epilimnion. Recently, George (1981) has noted chemical changes typical of nitrification in the hypolimnion of the south basin of Windermere. This lake is classified as meso to eutrophic and ammonia accumulates in the hypolimnion due to high productivity and not morphometric characteristics.

The concentrations of ammonia, at which nitrification becomes the dominant process, show no consistancy between the different lakes. However it is possible to generalize that ammonia, which accumulates under aerobic conditions in the hypolimnia of certain lakes, will be subject to nitrification if oxygen concentrations do not become limiting.

The inability to detect nitrification rates 1 m above the sediments in Grasmere is unusual, particularly as they were apparent at 5 m above the sediments and in the detailed survey of Grasmere in 1977. This could be due, in part, to infrequent sampling which could well have missed phases of nitrification which occurred between two sampling dates. A further possibility is the interaction between the  $^{14}\text{C}$ -bicarbonate added, the incubation time and the nitrifying activity of the sample involved, when using this method of estimating nitrification rates. The basis of the method is to detect differences, in the  $\text{CO}_2$  uptake, between N-Serve treated and untreated samples. The magnitude of this difference will depend on the three factors mentioned above. With a constant

nitrifying activity and incubation time a larger difference would be expected between samples which received  $20 \mu\text{Ci } ^{14}\text{C-HCO}_3^{2-}$  than between those receiving only  $5 \mu\text{Ci } ^{14}\text{C-HCO}_3^{2-}$ . Similarly given constant nitrifying activity and  $^{14}\text{C-HCO}_3^{2-}$  additions the expected difference should increase with increasing incubation periods. Therefore with three hours incubation, in the presence of  $5 \mu\text{Ci } ^{14}\text{C-HCO}_3^{2-}$  the nitrifying activities in these lakes, at certain depths and times of the year cannot be detected. Somville (1978) reported that incubation periods of 3 to 6 hs with  $25 \mu\text{Ci } ^{14}\text{C HCO}_3^{2-}$  added to water samples from the Scheldt estuary, measured detectable nitrification rates (range  $0-14 \mu\text{g N l}^{-1} \text{ h}^{-1}$ ) indicating these conditions gave adequate sensitivity. The sensitivity must also be related to the contribution, if any, of organic carbon to the growth of these lithotrophic organisms as discussed on page 142 and 143.

With the exception of Buttermere, all the lakes showed greater nitrification rates than the maximum rate measured in the detailed survey of Grasmere in 1977. As Buttermere is the most oligotrophic of the lakes studied the low rates of nitrification could be due to low ammonia concentrations present in the lake. However, Windermere did not show substantially greater net ammonia concentrations although gross concentrations could be large with a rapid turnover time. This hypothesis is supported by the consistently high nitrate concentrations found in this lake. The ability to detect nitrification rates more frequently in Brotherswater and Derwentwater, under the same experimental conditions, is puzzling. Although there is insufficient data available to classify the trophic status of Brotherswater it is known that increasing dissolved organic carbon concentrations are associated with increasing eutrophication. It is tempting to speculate that in the more eutrophic lakes organic carbon contributes more to the carbon budget of the lithotrophic nitrifiers and so results in the decreased sensitivity of the  $^{14}\text{C} / \text{N-Serve}$  method as used. Without further data this hypothesis

is purely speculative but in view of the present results indicating the importance of nitrification, and the interest recently displayed in this method (Fenchel and Blackburn, 1979) this is one area of research into nitrifier ecology which requires further attention.

The ability, in some instances, to associate nitrifying activity with the nitrification patterns of inorganic nitrogen concentrations, provides further evidence of the occurrence of planktonic nitrification. The importance of this process in Grasmere has been described and but for oxygen limitation, the process could be even more significant. In mesotrophic lakes where reduced nitrogen is in steady, but not excessive, supply and oxygen does not become limiting, nitrification could be extremely important. The regular detection of nitrification rates in the early season, not associated with typical chemical changes, in conjunction with the above results, attach a relevance to planktonic nitrification which has been overlooked in the past.

### 5.5 Nitrification in Grasmere sediments

Each sediment site in Grasmere (Section 4.2) was sampled at least two times during the period June to September 1978. The redox potentials, ammonia and nitrate concentrations in the interstitial waters were measured and rates of nitrification estimated at 0.5 cm intervals, to a depth 1.5 cm into the sediment cores. The results, expressed as mean values for corresponding layers in duplicate cores, are shown in Figures 5.17, 5.18, 5.19, 5.20 and 5.21 for sites A, B, D, E and E1 respectively.

#### 5.5.1 Redox potentials

The redox potential of a freshwater sample is relatively insensitive to changes in oxygen concentration, with only a 30 mV decrease as oxygen decreases from 10 to 0.1 mg l<sup>-1</sup> (Stumm and Morgan, 1970). Although there

are many errors inherent in the measurement of the redox potential in natural systems (for a full discussion see Stumm and Morgan, 1970), which preclude a quantitative interpretation, the appearance of a redox discontinuity layer in the water column usually indicates a change in bacterial metabolism in the sediment, from the use of oxygen to alternative electron acceptors. In sediments the measured redox potential is more difficult to define in relation to oxygen concentrations, due to the greater concentrations of chemical species (C, N, O, S, Fe and Mn containing compounds) and their ability to participate in redox processes. These can artificially poise the measured redox potential. However, some workers have suggested that a redox potential of +350 mV indicates oxygen disappearance (Keeney, 1973; Revsbech et al, 1980). Care must be taken, however, in the interpretation of measured redox values when the chemical composition of the environment is unknown. In the present work a redox potential of +200 mV was considered to be the absolute lower limit for oxidative processes e.g. nitrification to occur. This corresponds to the redox potential measured by Mortimer (1941-42) associated with the appearance of ferrous iron, but may be too low when compared to the findings of Revsbech et al (1980) who measured oxygen concentrations and redox potentials independently in marine sediments. When zero oxygen concentrations were recorded the corresponding redox potential was between +350 and +400 mV. Jones (1979) suggested that the measurement be referred to as Eh, and not redox potential, as the former term does not imply the involvement of specific redox couples.

In the present work the electrode system used to measure Eh is probably accurate only to within 50 mV of the actual value (Jones, 1979) and corrections for pH and total ionic concentration were not made. In presentation of the Eh measurements for the different sites (graph 1 of Figures 5.17 to 5.21) the areas of sediment with an Eh of +200 mV or more have been hatched to aid interpretation.

Figures 5.17 to 5.21    Depth distribution diagrams, on the dates specified, of redox potential, ammonia and nitrate concentrations in the interstitial waters and rates of nitrification in the sediments at five sampling sites on Grasmere.

Figure 5.17 Sediment: Site A (depth of overlying water, 21.5 m)

- 1) Redox potential (mV)
- 2) Ammonia concentration ( $\mu\text{g N l}^{-1}$  interstitial water)
- 3) Nitrate concentration ( $\mu\text{g N l}^{-1}$  interstitial water)
- 4) Nitrification rate ( $\mu\text{g NO}_3\text{-N produced g}^{-1}$  dry wt sediment  $\text{day}^{-1}$ ).

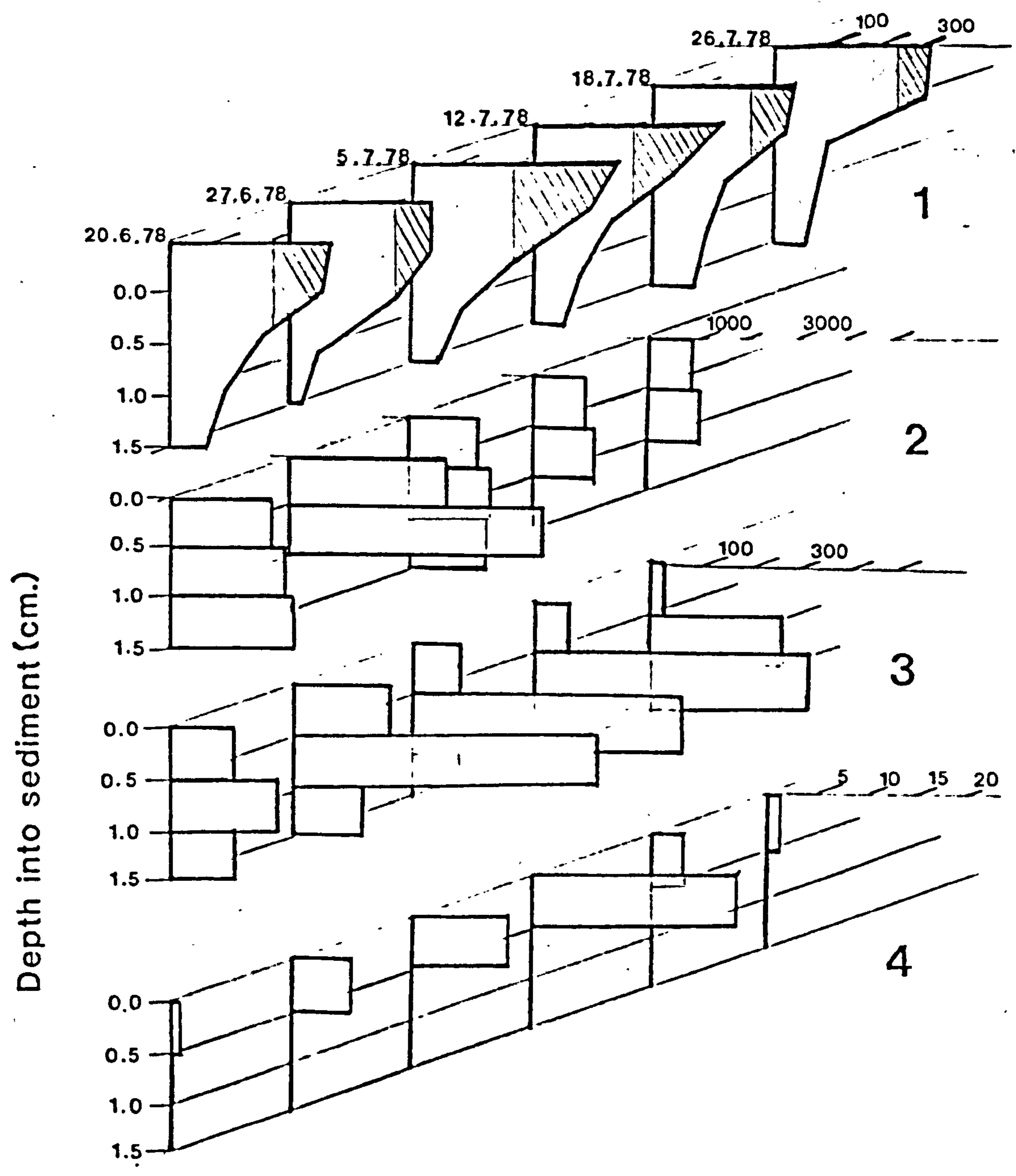




Figure 5.18 Sediment: Site B (depth of overlying water 16 m)

- 1) Redox potential (mV)
- 2) Ammonia concentration ( $\mu\text{g N l}^{-1}$  interstitial water)
- 3) Nitrate concentration ( $\mu\text{g N l}^{-1}$  interstitial water)
- 4) Nitrification rate ( $\mu\text{g NO}_3\text{-N produced g}^{-1}$  dry wt. sediment  $\text{day}^{-1}$ )

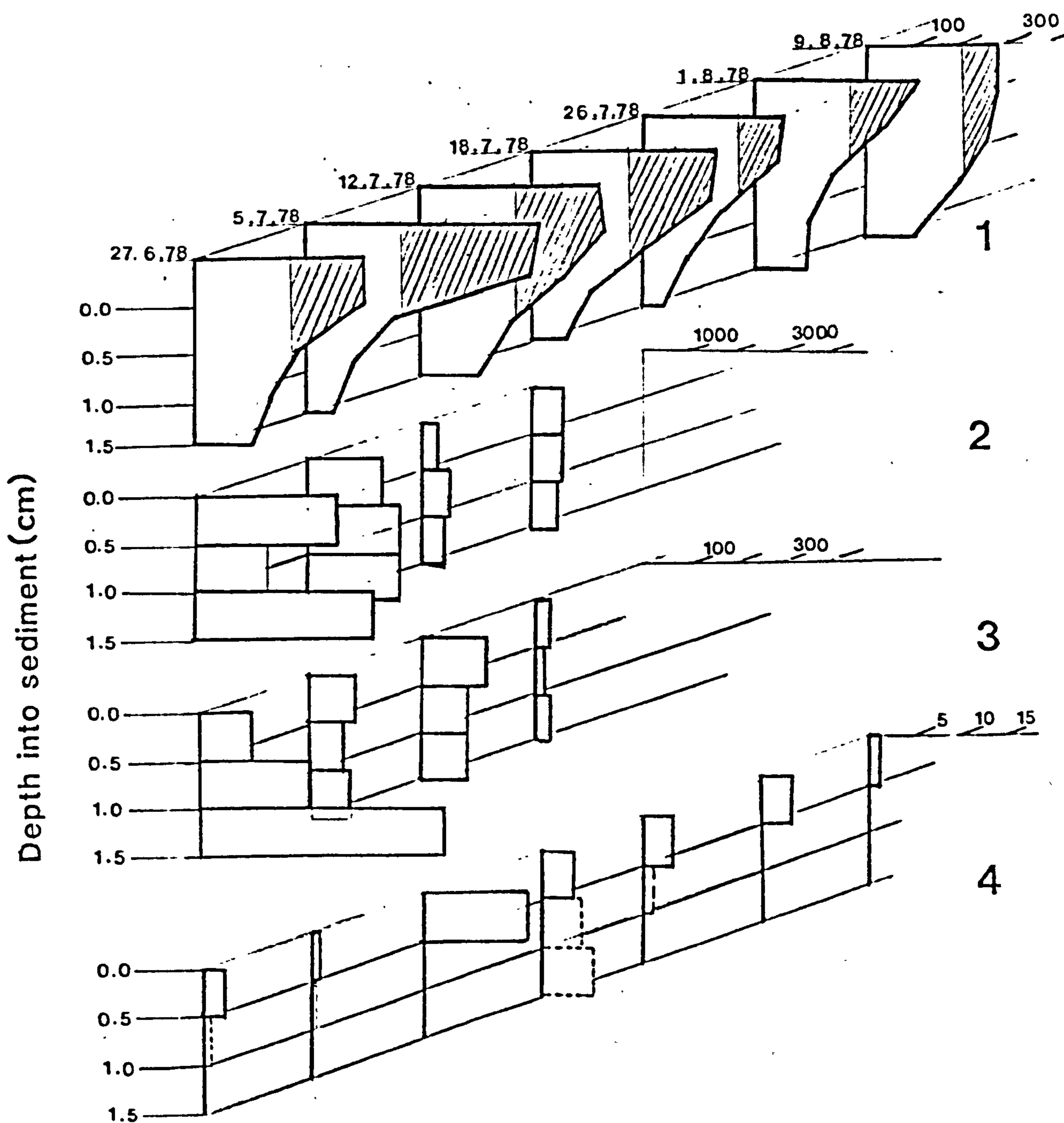


Figure 5.19 Sediment: Site D (depth of overlying water, 5 m)

- 1) Redox potential (mV)
- 2) Ammonia concentration ( $\mu\text{g N l}^{-1}$  interstitial water)
- 3) Nitrate concentration ( $\mu\text{g N l}^{-1}$  interstitial water)
- 4) Nitrification rate ( $\mu\text{g NO}_3\text{-N g}^{-1}$  dry wt. sediment.  $\text{day}^{-1}$ )

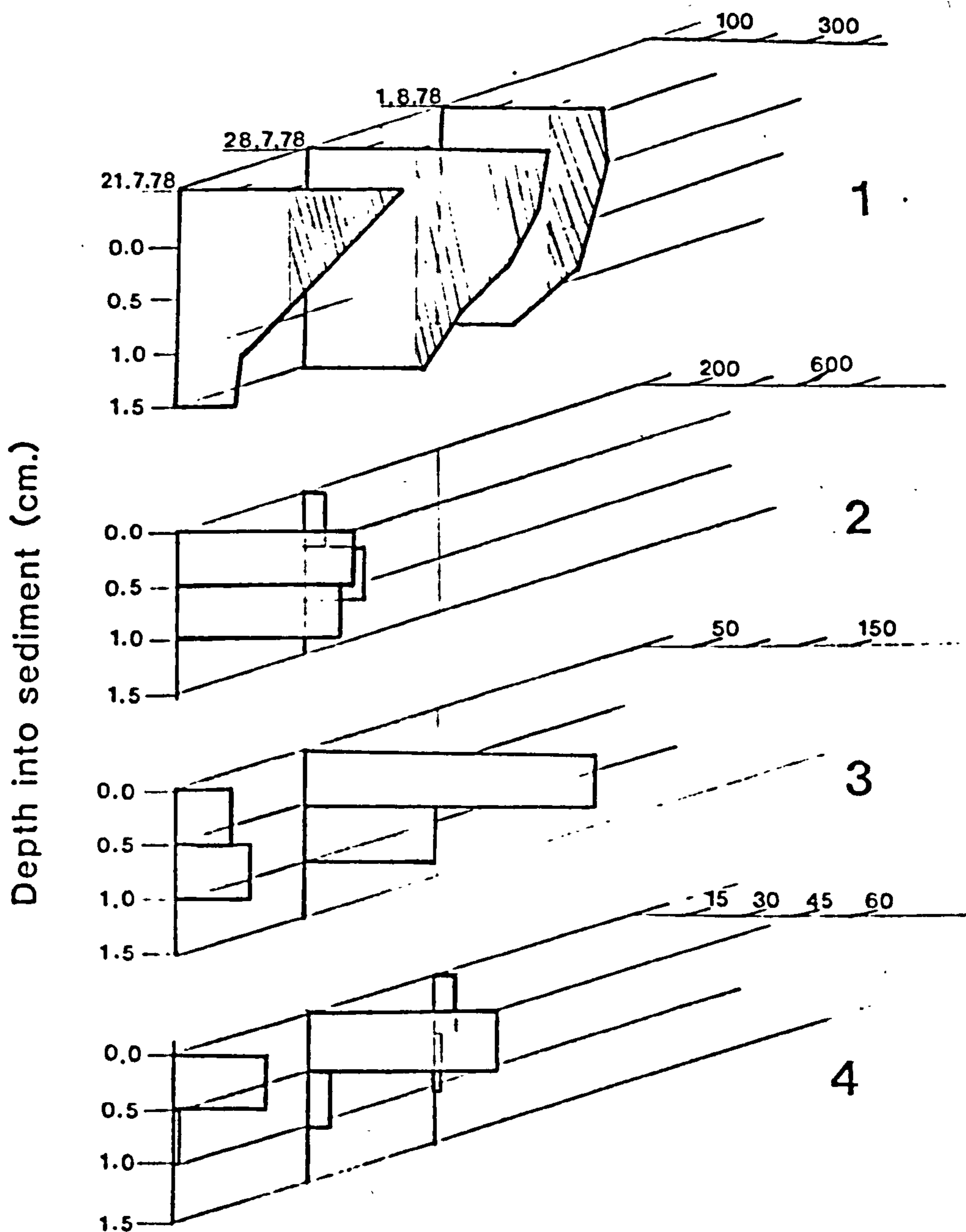


Figure 5.20 Sediment: Site E (depth of overlying water, 1 m)

- 1) Redox potential (mV)
- 2) Ammonia concentration ( $\mu\text{g N l}^{-1}$  interstitial water)
- 3) Nitrate concentration ( $\mu\text{g N l}^{-1}$  interstitial water)
- 4) Nitrification rate ( $\mu\text{g NO}_3\text{-N produced g}^{-1}$  dry wt. sediment  $\text{day}^{-1}$ ).

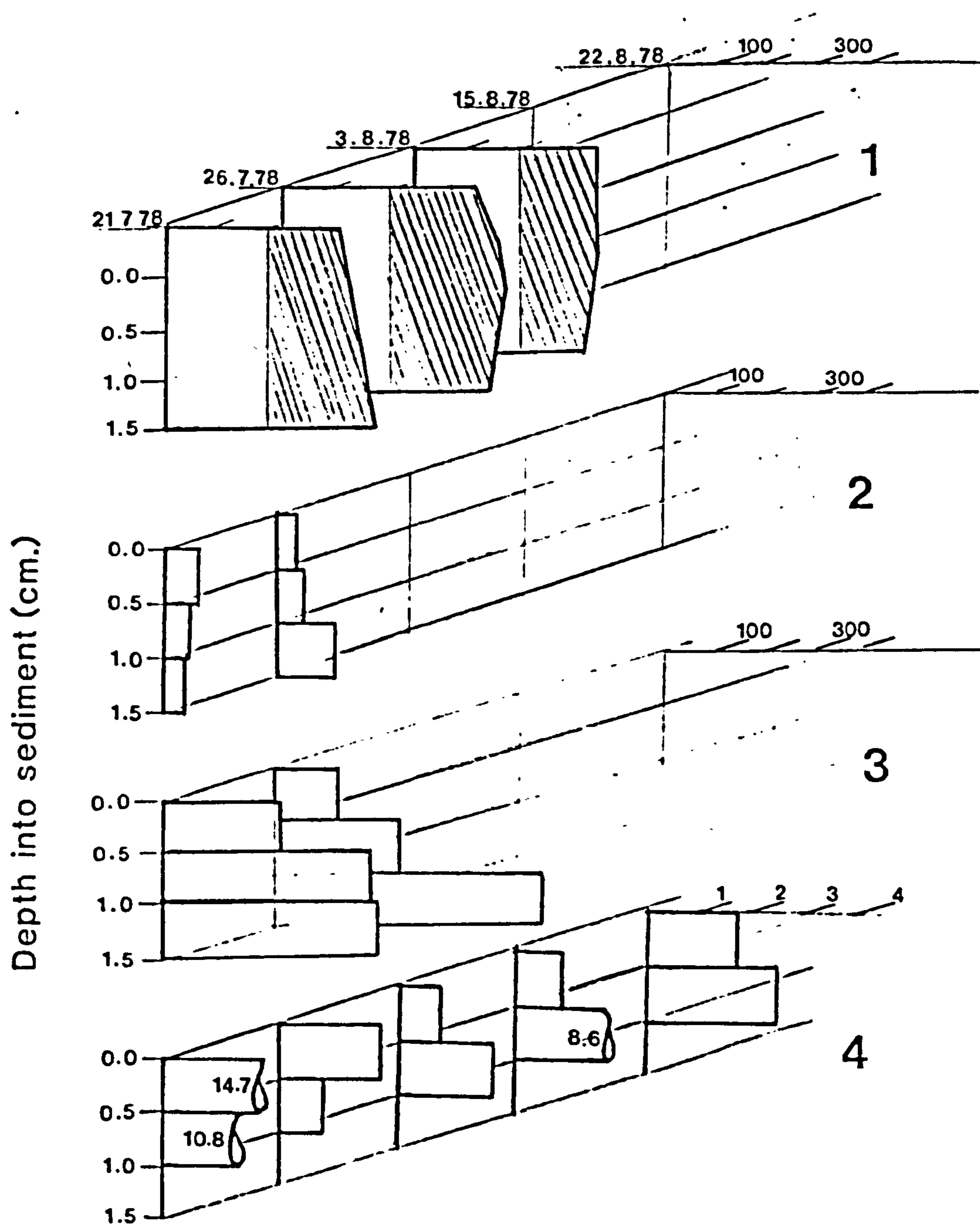
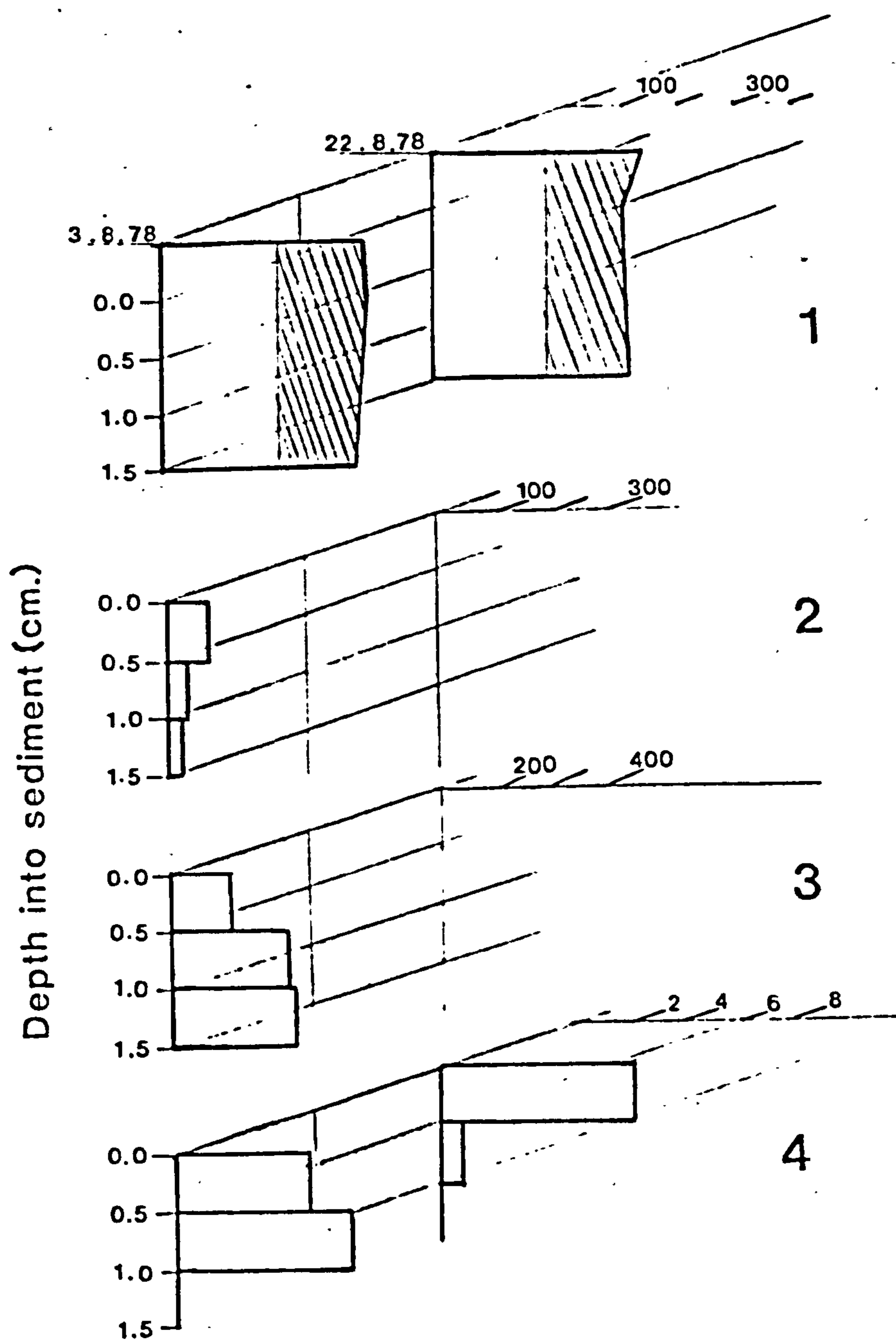


Figure 5.21 Sediment: Site E<sub>1</sub> (depth of overlying water, 1 m)

- 1) Redox potential (mV)
- 2) Ammonia concentration ( $\mu\text{g N l}^{-1}$  interstitial water)
- 3) Nitrate concentration ( $\mu\text{g N l}^{-1}$  interstitial water)
- 4) Nitrification rate ( $\mu\text{g NO}_3\text{-N produced g}^{-1}$  dry wt sediment day<sup>-1</sup>)



With the exception of site C all sediments showed little change in Eh between the overlying water and the sediment-water interface. At the time of sampling the interface Eh at site C was less than +200 mV and therefore further samples were not taken. This agrees with the findings of Jones et al (1980) who observed the most rapid deoxygenation of overlying waters at this site due, presumably, to its enrichment by the main river inflow.

The measured Eh gradients at the other five sites indicated differences between sediment types. At sites A and B (21.5 and 16 m deep respectively) a steep discontinuity layer was observed between the interface and 1.0 cm depth such that measurements greater than +200 mV were rarely recorded 0.5 cm into the sediment. At site D (5 metres depth) the gradient was not as steep, or occurred at greater depths, and Eh's of +200 mV, and above, were measured 1.5 cm into the profile. The Eh at sites E and E<sub>1</sub> showed no difference between the overlying water and sediment 1.5 cm deep.

Although the full chemical nature of the sediments is unknown the measured Eh values can be related to oxygen concentrations in the overlying water. At sites A and B the overlying water at the time of sampling would have an oxygen deficit and therefore the Eh discontinuity layer would be expected to be close to the sediment surface. At site D, in the littoral zone, the overlying water, and possibly interstitial waters, would be well oxygenated due to photosynthesis and turbulence. Given that one of the factors determining the depth of oxygen penetration into the sediment is the overlying oxygen concentration, the Eh discontinuity layer would be expected to be deeper in these littoral sediments. Sites E and E<sub>1</sub> were sandy in nature and the absence of an Eh gradient could be a result of high overlying oxygen concentrations, sediment porosity and possibly benthic primary production.

### 5.5.2 Inorganic nitrogen concentrations

The results for the different sampling sites, presented as histograms for the separate 0.5 cm sediment depth layers, are summarized in graphs 2 and 3 in Figures 5.17 to 5.21. It was impossible to extract sufficient interstitial water for full chemical analysis from the deeper sediment layers and preference was given to alkalinity determinations.

Although the sensitivity and precision of the methods were not checked there is information on possible interferences available in the literature. The use of spongy cadmium metal as a nitrate reductant would be subject to errors (Davison and Woof, 1979). Downes (1978) reviewed and investigated possible interferences with the hydrazine-cupric ion reductant and improved the performance by addition of  $Zn^{2+}$  ions to saturate compounds capable of providing ligands which could, otherwise, bind and remove cupric ( $Cu^{2+}$ ) ions from solution. There is little information on interferences with the Indophenol method of ammonia determination (adapted from Crooke and Simpson, 1971) except that both mercury and sea water affect colour development (M. Gibbs pers. comm.).

#### 1. Ammonia

This was the dominant inorganic form of nitrogen at sites A, B and D where organic rich sediments were found. At sites A and B the ammonia concentrations were at least an order of magnitude higher than the concentration of nitrate, but the difference was considerably less at site D (2 to 4 times). The lowest ammonia concentrations (0 to 100  $\mu g N l^{-1}$ ) were found at sites E and  $E_1$ . Generally speaking, at individual sites ammonia concentrations showed no change, or increased, with depth into the sediment. Where there was sufficient data for comparison (sites A and B) ammonia concentrations tended to decrease throughout the study period.

## 2. Nitrate

Nitrate was the dominant inorganic nitrogen species at both sites E and E<sub>1</sub>, being two to three times the ammonia concentration. Nitrate concentration increased with depth at both these sites. The concentrations at the other sites were variable and it is not clear if these variations were real or due to methodological errors. At site A (21.5 m deep) nitrate concentration maxima were regularly observed between 0.5 and 1.0 cm depth. At site B (16 m) the concentration of nitrate either increased with depth or was constant throughout the profile. At sites A and B the nitrate concentration in the overlying water exceeded that in the sediments and therefore there would be a diffusion gradient into the sediment. In the littoral zone (sites D, E and E<sub>1</sub>) the nitrate gradient would be zero or reversed. The observed concentrations would depend on the rates of the biological processes utilizing and producing nitrate, and any diffusion which occurred.

### 5.5.3. Nitrification rates

In the calculation of these rates, from the formula given in section 4.7.2, it was assumed that the total inorganic carbon content of the interstitial water, and not the total inorganic carbon of the sediment (i.e. liquid and solid phase) represented the total CO<sub>2</sub> available. This assumption was thought to be valid due to the slow rate at which equilibrium is attained between the solid and liquid phase inorganic fractions (W. Davison pers. comm.) compared with the incubation time of 3 h. The total CO<sub>2</sub> determination, as used, could result in underestimates due to CO<sub>2</sub> exchange with the atmosphere during interstitial water extraction and preparation for alkalinity determinations.

The calculated carbon uptake rates for the sediments within treatments (i.e. alcohol or N-Serve) were highly variable; so much so that rarely were differences between N-Serve and control treatments significant.

However, the results did compare well with other environmental variables measured, particularly Eh, and are presented in graph 4 of Figures 5.17 to 5.21 as  $\mu\text{g N oxidized g}^{-1} \text{ dry wt day}^{-1}$  (calculated from the product of N-Serve sensitive  $\text{CO}_2$  uptake and the factor 9.01). Although the measured Eh 0.5 cm into the sediment at sites A and B precluded the possibility of the occurrence of nitrification, estimates were made as the process could occur in the oxidised first few millimetres of sediment.

At site A, as expected, nitrification was only detected in the top 0.5 cm of sediment. The maximum rate measured was  $15 \mu\text{g N g}^{-1} \text{ dry wt day}^{-1}$  and the mean rate over the sampling period was  $5 \mu\text{g N g}^{-1} \text{ dry wt day}^{-1}$ . Similar results were obtained from site B although on four occasions N-Serve sensitive  $\text{CO}_2$  uptake was noted in deeper sediments (shown as dotted lines in graph 4, Figure 5.18). This was unusual as the Eh, measured in these deeper sediments, precluded the occurrence of nitrification. As discussed above, the measured Eh may not indicate the actual condition of the sediments. It is possible that the in situ Eh was disturbed whilst handling the sample. Billen (1976) has shown that marine sediments rapidly regain in situ Eh values, during incubation, after similar handling techniques. However, similar results at site A, to those found at site B, would be expected which was not the case. The possible involvement of microorganisms other than the nitrifying bacteria, in N-Serve sensitive inorganic carbon uptake will be considered in the discussion to follow. The surface sediment at site B had maximum and mean nitrification rates of 8.3 and  $2.7 \mu\text{g N g}^{-1} \text{ dry wt day}^{-1}$  respectively. The corresponding values for the supposedly anoxic deeper layers were 4.0 and  $1.8 \mu\text{g N g}^{-1} \text{ dry wt day}^{-1}$ .

In the littoral sediments (sites D, E and  $E_1$ ) the measured Eh indicated oxidising conditions to 1.5 cm depth into the sediments. Nitrification at site D was only detected in the surface 1.0 cm. At



sites E and E<sub>1</sub> estimates of nitrification rates, below 1.0 cm, were not attempted because of the consolidated nature of the deposits and consequent difficulty of handling. At site D the surface sediments always showed greater activity than the sediments 0.5 to 1.0 cm deep; the corresponding maximum rates being 39 and 4.5  $\mu\text{g N g}^{-1}$  dry wt day<sup>-1</sup> respectively. Sites E and E<sub>1</sub> showed considerable variability of activity with depth into the sediments, sometimes being greater 0.5 to 1.0 cm deep, sometimes less. The maximal rates measured for these sediments were 14.7 and 5  $\mu\text{g N g}^{-1}$  dry wt day<sup>-1</sup> for sites E and E<sub>1</sub> respectively. The corresponding mean values being 4.2 and 3.5  $\mu\text{g N g}^{-1}$  dry wt day<sup>-1</sup>

#### Discussion

The literature was reviewed for possible interferences with the chemical determinations. The considerable variation noted in results in the present study, particularly for profundal sediments, where environmental conditions (Eh and temperature) remained fairly stable, indicated that interference might be occurring. The use of internal standards, for checking the performance of the chemical determinations, should be used in future work.

Although confidence limits could not be calculated for the estimated nitrification rates from the different sites certain generalizations can be made regarding their gross magnitude. The highest rates were consistently measured in the littoral sediments at site D. The maximum rate (46  $\mu\text{g N g}^{-1}$  dry wt day<sup>-1</sup>) was approximately three times the maximum rate measured at all the other sites. Littoral sediments might be expected to have greater activities due to increased temperatures and possibly increased aeration. However, this hypothesis does not explain all the observations. Sites E and E<sub>1</sub>, which were also in the littoral zone, had a maximum measured rate (14.7  $\mu\text{g N g}^{-1}$  dry wt day<sup>-1</sup>) very

similar to the maximum rate measured in profundal sediments ( $15 \mu\text{g N g}^{-1} \text{ dry wt day}^{-1}$ ) even though there were temperature and oxygen concentration differences. However, activity in these littoral sites did extend deep into the sediments whilst activity in the profundal zone was restricted to surface sediments. A possible explanation for the low rates at the littoral sites E and E<sub>1</sub> was the low ammonia concentrations found in these deposits which never exceeded  $100 \mu\text{g N l}^{-1}$  interstitial water. Nitrification in the water column, however, occurred at much lower ammonia concentrations.

Jones et al (1980) observed mean nitrate reductase activities in the top 1.5 cm of Grasmere sediments of between  $2$  and  $22 \text{ mg NO}_3\text{-N l}^{-1} \text{ h}^{-1}$  during a survey of different sites in 1978. Conversion of these values to units used in the present study (assuming the sediment contains 5% dry weight and 1 ml weighs 1 g) gives values of 2.4 to 10.56  $\text{mg NO}_3\text{-N g}^{-1} \text{ dry wt day}^{-1}$ . These are approximately three orders of magnitude (1000X) greater than the maximum nitrification rate measured. However, the method of estimating nitrate reductase activity will give maximal potential values as the enzyme is extracted and therefore cell wall barriers are removed, the reaction mixture is completely reduced (chemically), and the reaction is unlimited in nitrate concentration. An estimate of in situ nitrate reductase activity would possibly be an order of magnitude lower (J.G. Jones pers. comm.). This still gives nitrate reductase activities two orders of magnitude greater than the maximum measured nitrification rate. If these two processes were active at these rates in situ any nitrate produced should be removed from the system as nitrate reduction in sediments appears to follow first order reaction kinetics in respect to nitrate concentration (Laudelot, 1978). The rate of removal would, however, be dependent on the value of the half saturation constant ( $k_m$ ) of nitrate reductase for nitrate. Although nitrate is never the dominant inorganic form in

the rich organic sediments the present results do indicate substantial concentrations. This suggests, as for the water column, actual nitrification rates may be underestimated by this method.

Further evidence for nitrate reduction being the primary factor controlling nitrate concentrations in the sediments comes from observations on the carbon to nitrogen ratios for sediment sites A and E shown in Table 5.8. The high C:N ratio for site A sediments would be expected as these have already been described as typical organic rich deposits. The large organic fraction would serve as hydrogen donor for nitrate reducing bacteria. It is not clear if the high ammonia concentrations typical of these sediments is due to ammonification or ammonia being an end product of dissimilatory nitrate reduction. The low C:N ratios observed for site E sediments indicate a carbon limitation for all heterotrophic activity. However, the failure to measure redox potential discontinuity layers in these sediments may preclude the occurrence of nitrate reduction. Both these factors may contribute to the dominance of nitrate in the inorganic nitrogen fraction within these sandy sediments.

Converting the maximum estimated nitrification rate, measured in surface sediments at site A to activity per ml (assuming that this sediment is 5% dry weight and 1 ml sediment weighs 1 g), a value of approximately  $1.0 \mu\text{g N ml}^{-1} \text{ day}^{-1}$  is obtained. Comparison of this value with the highest estimated nitrification rate in the water column (which is at approximately the same temperature) shows that the sediment activity is approximately 150 times greater. Although confidence limits cannot be placed on the sediment estimates this observation is in agreement with other studies (Isirimah *et al*, 1976; Christofi, 1978). This corresponds to the general finding that numbers, and activity, of bacteria in freshwater sediments, are much greater than those found in the overlying water (Jones, 1979).

Table 5.8 Mean carbon to nitrogen ratios from different depths into the sediments of sampling sites A and E on Grasmere. (The mean is a result of duplicate C:N analyses on two sampling dates).

	Depth into sediments (cm)			
	Site A		Site E	
	0.0 → 0.5	0.5 → 1.0	0.0 → 0.5	0.5 → 1.0
C:N ratio	11.4 (0.5)	10.9 (0.7)	1.12 (0.29)	1.42 (0.42)

( ) standard deviation

The detection of N-Serve sensitive carbon uptake in sediments greater than 0.5 cm depth at site B was unusual. As discussed above this could be due to errors in the interpretation of, or changes in, the redox potential. However, Somville (1978) noted a strain of Desulphovibrio desulphuricans (a sulphate reducing bacterium) to be sensitive to N-Serve at concentrations known to inhibit nitrifying organisms (see Table 2.3). He attributed N-Serve sensitive carbon uptake, measured in anaerobic water samples from the Schledt estuary, to the activity of similar organisms. There is no information on the occurrence of this process in Grasmere although sulphate reduction is thought to become rapidly sulphate limited in freshwater sediments (J.G. Jones pers. comm.). Jones et al (1980) noted substantial methane release from the sediments of site B in July 1978. This indicated the activity of methanogenic bacteria in the sediments. As methane production is the reverse of methane oxidation, a process known to be sensitive to nitrification inhibitors (Hubley, 1975), it is possible that N-Serve inhibits methanogenesis. The methane producing bacteria assimilate CO<sub>2</sub> and they could contribute to N-Serve sensitive carbon uptake.

Care must be taken, in the application of the <sup>14</sup>C-bicarbonate-N-Serve method, for nitrification rate estimation, in sediments which show steep redox discontinuity gradients. Under these conditions oxidative and reductive processes can occur in close proximity. The sensitivity of sulphate reducing bacteria to N-Serve is well established and Rudd and Taylor, (1980) discuss possible complex interrelationships between these organisms and methanogenic bacteria.

## 5.6 Observations on the isolation of lithotrophic nitrifiers from the aquatic environment

### 5.6.1 Enrichment

Using the full strength mineral salts medium of Skerman (1967), considerable difficulty was experienced in the culture of both phase I and II nitrifiers from the water column of Grasmere, even during the period of active nitrification. However, with an increase of inoculum volume, activity of the phase I organisms was finally detected. Having established, through the use of N-Serve, that the observed increases in hypolimnetic nitrate concentration were due to a totally chemolithotrophic process one would expect substantial nitrifier populations to be present. Belser and Schmidt (1980) present data, based on culture work, and therefore not directly applicable to field situations (see Belser, 1979), from which an overall average oxidation rate of  $0.199 \times 10^{-6} \mu\text{g N oxidised hr}^{-1} \text{ cell}^{-1}$  for phase I bacteria can be calculated. Assuming a field nitrate production value of  $7 \mu\text{g N l}^{-1} \text{ day}^{-1}$  and that all nitrite produced is oxidised to nitrate, a population of  $1.5 \times 10^6$  phase I organisms  $\text{l}^{-1}$  should be present (this number will increase with decreasing oxidation rates per cell, likely to be found under in situ conditions). Therefore a small inoculum (i.e. 1 ml) should result in a successful enrichment. The fact that such attempts failed suggested that culture conditions were inadequate. The observation that activity was detected with increasing inoculum volume indicated that dilution of the medium produced a favourable growth environment. A series of experiments were performed in which the basal mineral salts medium (see Section 4.9) was diluted to obtain final basal media concentrations of 1, 10, 20 and 40% (V/V with double distilled water). Substrate, as  $(\text{NH}_4)_2\text{SO}_4$ , was added to give final nitrogen concentrations of 1 to 400  $\text{mg N l}^{-1}$  (which covered the range used in a majority of media, Table 2.2). After adjustment of the pH

to 7.6 triplicate samples of medium were inoculated with 10 ml aliquots of lakewater and incubated as described in Section 4.9. Spot tests for the appearance of nitrite were made at 24 or 48 hour intervals; the presence of ammonia, particularly at the lower concentrations, was determined at weekly or fortnightly intervals. The results, for samples taken at different times of the year, expressed as the time required, in days, for detectable quantities of nitrite to appear in separate flasks, are shown in Table 5.9. The dates reported cover three years and do not relate to observations within one year. From this table it can be seen that either the low substrate concentration or low basal medium concentrations, or sometimes a combination of both, consistently enrich for the phase I organisms most rapidly. The higher substrate and basal media concentrations often show a complete lack of activity or extremely long lag periods. It is possible that, at certain times of the year, there were no nitrifying organisms present in the 10 ml inoculum used. However detection of activity in some flasks, in all experiments, precludes this possibility. The failure to obtain growth and/or activity must be due to unfavourable culture conditions. The reproducibility of the results was also of interest. Only the lowest basal medium and substrate concentrations consistently showed activity in all these flasks, although at times, there was considerable variability in the incubation time required. This is important with regard to the assumptions underlying M.P.N. methodology, in which replicate inoculums from a set dilution, are required. The medium must be able to support the growth of the organisms if present in the inoculum to prevent inaccurate interpretation of results. Clearly at the higher concentrations of basal medium and substrate this assumption will not be valid.

Although this experiment was designed to measure the nitrification potential of samples the results obtained cannot be interpreted in such

Table 5.9 The effect of varying concentrations of basal mineral salts medium, and substrate (ammonia - N) concentration, on the enrichment of ammonia oxidising chemolithotrophic bacteria from lake water.

Table shows the incubation time, in days, before nitrite was detected.

21st December															
Substrate concentration mg N L <sup>-1</sup>	Medium strength			1%			10%			20%			40%		
	4	22	15	18	25	25	20	-	-	-	-	-	-	-	-
150	-	22	25	-	-	-	25	25	-	25	25	-	-	-	-
400	-	-	-	-	22	-	-	-	-	-	-	-	-	-	-

20th March															
Substrate concentration mg N L <sup>-1</sup>	Medium strength			1%			10%			20%			40%		
	4	20	20	28	28	20	30	30	28	-	45	-	41	-	-
150	45	45	28	24	28	-	49	-	-	-	49	-	-	-	-
400	30	28	45	-	-	-	-	49	-	-	45	-	-	-	-

30th May															
Substrate concentration mg N L <sup>-1</sup>	Medium strength			1%			10%			20%			40%		
	4	14	25	17	-	-	25	-	-	-	-	-	-	-	-
150	28	-	28	-	31	-	-	36	-	-	-	-	-	-	-
400	-	28	-	-	-	-	-	-	-	-	-	-	-	-	-

- indicates no nitrite appearance during the incubation period.



Table 5.9 continued

9th July												
	1%			10%			20%			40%		
	4	12	12	14	22	—	—	19	—	—	—	—
150	—	22	—	—	—	—	48	—	—	—	—	—
400	—	—	—	—	22	—	41	41	41	—	—	—

18th September												
	1%			10%			20%			40%		
	4	10	10	10	19	19	19	19	22	19	22	19
150	19	31	31	42	42	—	42	—	19	—	—	—
400	—	—	42	—	—	—	—	—	42	—	—	—

a manner as the samples were taken over different years. The variability of incubation times required for activity to be detected could be a result of natural seasonal variations.

The medium formulation of 1% (V/V) basal medium and  $4 \text{ mg l}^{-1} \text{ N}$  (as  $(\text{NH}_4)_2\text{SO}_4$ ) was used, for M.P.N. estimates of phase I organisms, at 18 m depth in Grasmere, from May to July, 1980.

Culture conditions, for phase II organism enrichment, were not investigated.

#### 5.6.2 M.P.N. counts of phase I nitrifiers in Grasmere

One problem which arises from the use of low substrate concentrations in the growth medium is the low final concentration of nitrite (or nitrate) to be detected in the culture vessel. As defined, the initial substrate concentration in the medium was  $4 \text{ } \mu\text{g N ml}^{-1}$ . Assuming an average bacterial cell weight of  $10^{-13} \text{ g}$ , and an average nitrogen content of 14% (W/W), the substrate concentration allows considerable potential for bacterial growth. This growth could be lithotrophic or chemoorganotrophic using the organic substrates present in the inoculum or formed during the growth of lithotrophic organisms. Assuming 50% of the nitrogen is removed in this way the remaining concentration ( $2 \text{ } \mu\text{g ml}^{-1}$ ) is within the detection sensitivity of the chemical determinations used ( $\text{NH}_4^+$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ). However, nitrogen often completely disappeared from both the test tube and micro-titre dish systems. This was overcome in one of two ways. For the test tube systems ammonia substrate concentrations were replenished 14 days before the final analysis. Because of difficulties in handling, the replenishment of substrate in the micro-titre dish systems was impractical and so the initial substrate concentration was increased, which, as noted in Table 5.8 would reduce the enrichment efficiency of the medium. The micro-titre dish systems for water samples never gave meaningful

results and many combinations of positive scores were statistically invalid (see Rowe et al, 1977). This could possibly be the result of a combination of the lack of reproducibility associated with high substrate concentrations and a function of the small inoculum volume (50 or 150  $\mu$ l for the 1:4 or 1:2 dilution interval respectively). Therefore only results from the test tube systems ( $10^{-1}$  dilution interval, 5 replicates per dilution) are presented. Log. M.P.N. results, for the period May to July 1980, are presented in Table 5.9 with the corresponding nitrate concentrations throughout the nitrification phase. Although the data are limited, it can be observed that populations are present throughout the period of stratification but, more significantly, increase in late July. This increase is concomitant with the most rapid increase in nitrate concentration during the nitrification phase of this year. Sampling was stopped at the end of July as concentrations of oxygen, at 18 metres depth, fell below  $0.2 \text{ mg l}^{-1}$ . The range of phase I population estimates were between less than one organism  $\text{ml}^{-1}$  to 17 organisms  $\text{ml}^{-1}$ .

#### Discussion

The short lag periods noted in the isolation experiments (Section 5.6.1) were not apparent in the M.P.N. estimates. This may be because of the small number of organisms present in the inoculum and the time required to produce detectable quantities of nitrite. However, the tubes did show considerable variation in nitrite content after different incubation periods. For example, the count for the sample taken on 30th May was replicated and nitrite analysis performed after 54, 73, 81 and 103 days incubation. The M.P.N. counts obtained were 200, 200, 780 and 1,700 organisms per litre respectively. As the count was increasing it is quite possible that the reported count ( $1700 \text{ cells l}^{-1}$ ;

Table 5.10 M.P.N. counts (as  $\log_{10}$  bacteria  $l^{-1}$ ) of ammonia oxidising lithotrophic bacteria, and nitrate concentrations, at 18 m depth in Grasmere, 1980.

Date	Log. MPN $l^{-1}$ <sup>a</sup>	NO <sub>3</sub> -N ( $\mu$ g $l^{-1}$ )
30.5.80	3.23	
11.6.80	2.97	
23.6.80	2.65	
30.6.80	3.52	
5.7.80		505
7.7.80	3.69	520
9.7.80		591
11.7.80		635
14.7.80	2.89	606
16.7.80		682
20.7.80	3.85	
22.7.80		734
25.7.80	4.23	

<sup>a</sup> Standard error of log. MPN = 0.259

95% confidence limits = log MPN  $\pm$  0.52

Results are significantly different when log M.P.N. differs by 0.719.

log 3.23) is underestimated. This incubation time caused considerable practical difficulties and clearly there is a requirement to improve the culture conditions.

Many of the tubes recorded as positive for ammonia oxidising activity contained nitrate as the end product. If this spot test had not been included false negative scores would have been recorded. The population estimates cannot account for the observed increases in nitrate concentration when activities per cell, measured in culture, are used for calculation. As the nitrate accumulation, during the nitrification phase, has been demonstrated to be sensitive to chemolithotrophic nitrification inhibitors (Table 5.1) the M.P.N. method as used, although detecting appropriate population maxima must underestimate the actual population. I have criticised the use of such values obtained from cultures for field calculations and therefore consider that it is impossible to make any quantitative assessment of the counting efficiency of the medium. Clearly there is room for the application of fluorescent antibody techniques in this environment.

#### 5.6.3 M.P.N. counts of lithotrophic nitrifiers in Grasmere sediments

It is possible that, due to the nature of the environment, nitrifiers from the sediments tolerate greater concentrations of substrate or basal medium. However, in determining the number of organisms similar criteria to those used for the water samples were employed. Sole use of the micro-titre dishes entailed the use of greater substrate concentrations for reasons mentioned above. A substrate concentration of 40 mg N l<sup>-1</sup> was used which resulted in a concentration of 6 µg N in each culture well. Considerable difficulties were however encountered and these are illustrated in Plate 1. It can be seen that nitrite is only detected (the dark wells) in 3 of the 8 replicates at a dilution of 2<sup>-6</sup>; nitrate spot tests were also negative. The absence of nitrite, or

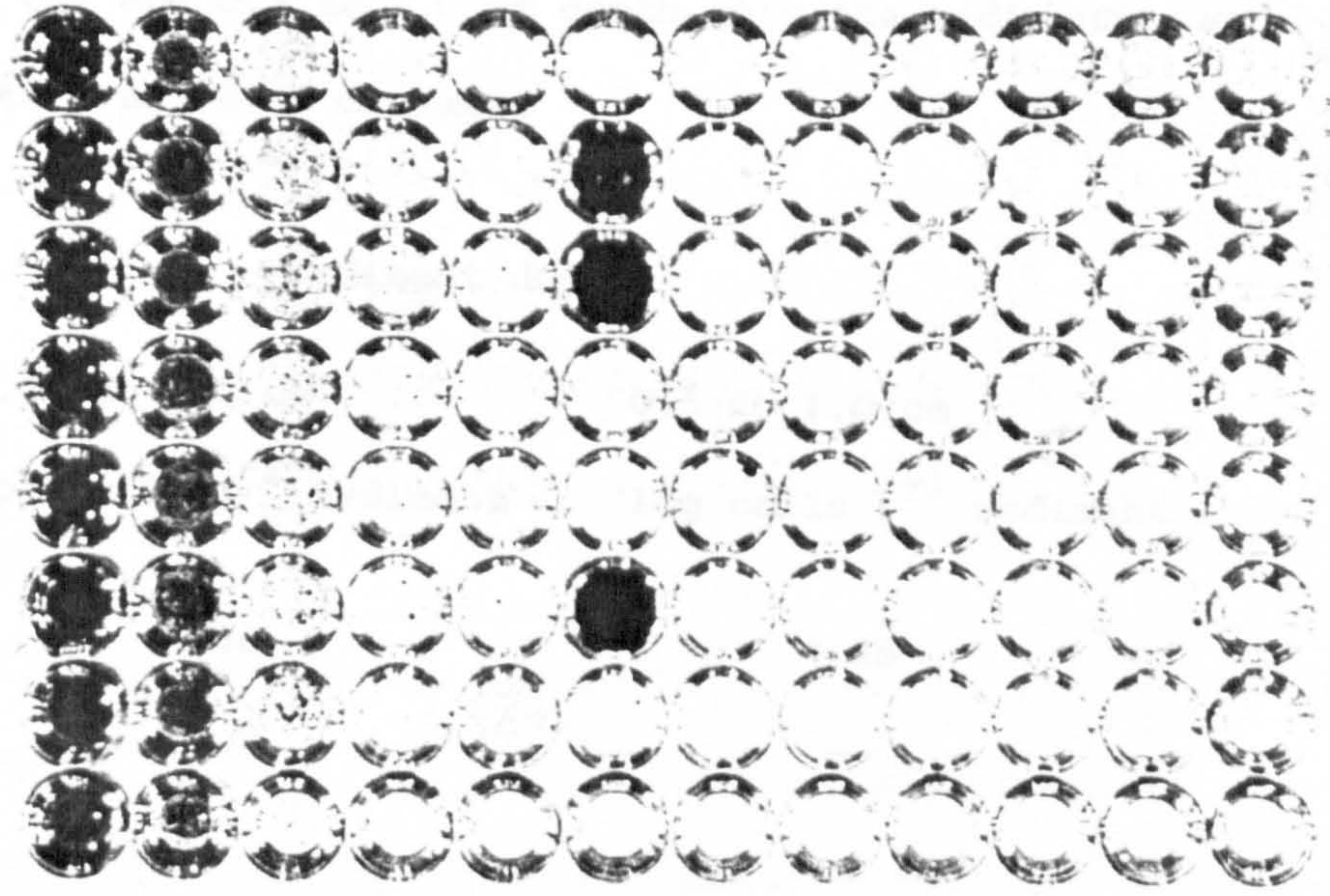
nitrate, in dilutions up to and including  $2^{-5}$  indicates that these nitrogen compounds have been removed from the systems (the presence of nitrite at a dilution of  $2^{-6}$  indicates that ammonia oxidisers must be present at lower dilutions). Heterotrophic uptake could account for some of this but theoretically a concentration of  $6 \mu\text{g N}$  could sustain a population of approximately  $10^9$  bacteria which, obviously, was not attained in the present work. Release of nitrous oxide gas has been observed in ammonia oxidising cultures (Ritchie and Nicholas, 1972) although the contribution that this might make in this case is unknown. Because the micro-titre dishes were enclosed, to reduce evaporation during incubation, oxygen may have become limiting and nitrite (or nitrate) reduction, to  $\text{N}_2$ , could occur. The adsorption of inorganic nitrogen forms to the plastic of the culture vessel cannot be discounted.

However, to estimate an M.P.N. count for results like those shown (Plate 1) the following assumption was made. The positive scores (based on nitrite production) at three successive dilutions, using Plate 1 as an example, were taken as  $8(2^{-5})$ ,  $3(2^{-6})$  and  $0(2^{-7})$ . Using the M.P.N. tables presented by Rowe et al (1977) an M.P.N. for this sequence of scores of  $282 \text{ bacteria ml}^{-1}$  is obtained.

This problem was encountered in all attempts to count phase I organisms in sediment deposits. However, using the above method the counts shown in Table 5.11 were obtained from the sediments at sites A and B in Grasmere. Counts for the month of July are presented to allow comparison with counts from the water column (Table 5.9) at the same time of year. Although the data are limited, and the samples taken in different years, the counts are equal to, or greater than the maximum counts obtained from the water column. Considering the imprecise nature of the M.P.N. estimate it is apparent that the counts of phase I nitrifiers in sediments exceed those in the water column. This accounts

Plate 5.1 Typical M.P.N. count analysis, in micro titre dish systems, for nitrite production by phase I nitrifiers in sediment. The dilution increases from  $2^{-1}$  to  $2^{-12}$  from left to right. The dark colour at dilutions of  $2^{-1}$  and  $2^{-2}$  is due to sediment in the wells.

Plate 5.2 Typical M.P.N. count analysis, in micro titre dish systems, for nitrite removal and nitrate production by phase II nitrifiers in sediments. Dilution increases from  $2^{-1}$  to  $2^{-12}$  from left to right. The dark colour at dilutions of  $2^{-1}$  and  $2^{-2}$  is due to sediment in wells.



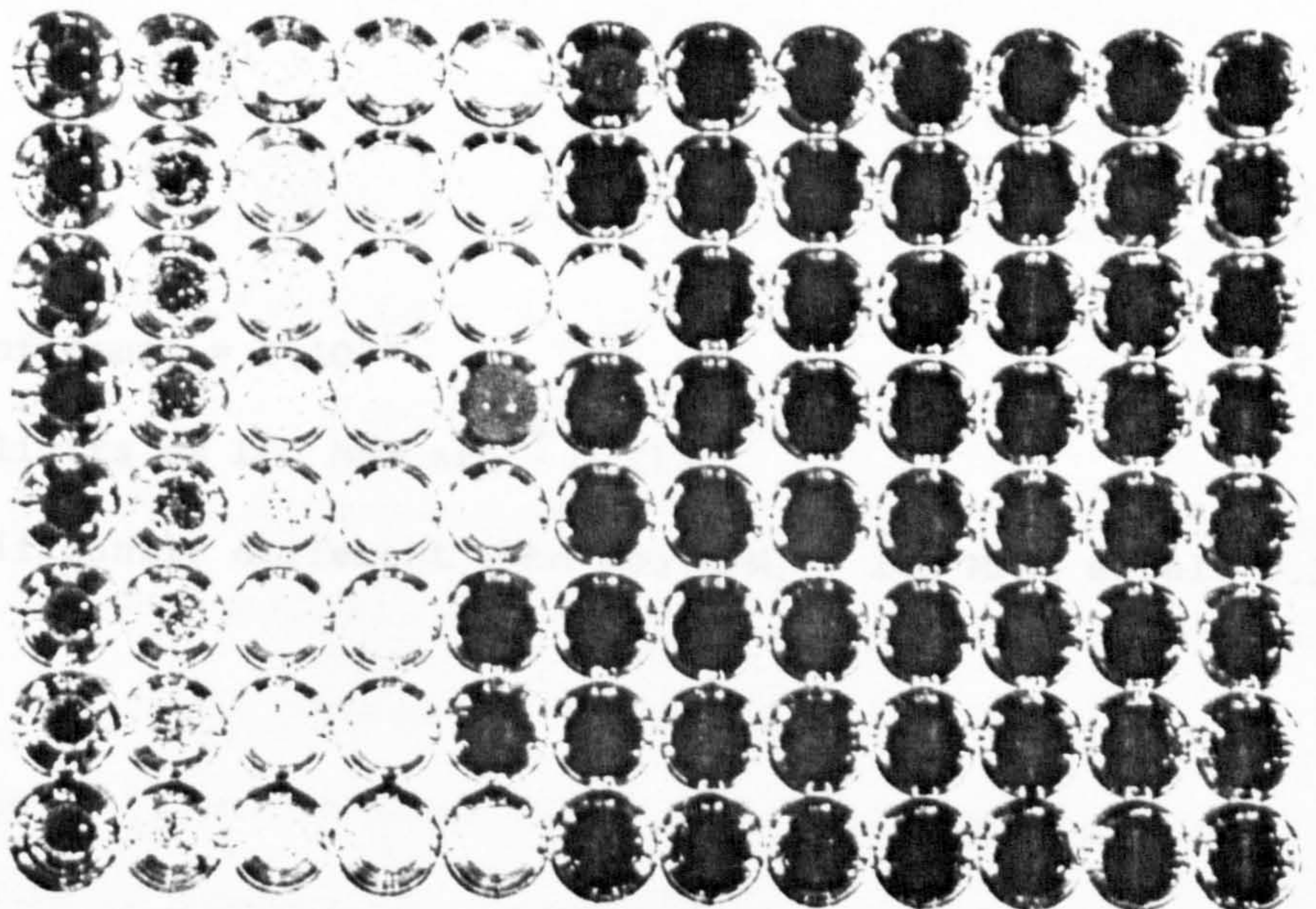
Relative depth

0.0 to 0.3 m

0.3 to 1.0 m

log cells  $10^4$  surface

log cells  $10^4$  surface



Standard error

95% confidence

Counts are signal



Table 5.11 M.P.N. counts of ammonia oxidising chemolithotrophic bacteria (as log bacteria  $l^{-1}$  wet sediment), at 0 to 0.5 cm, and 0.5 to 1.0 cm depth into the sediments, at Sites A and B on Grasmere

<u>SITE A</u>	Sediment depth	
	Date	
	0.00 to 0.5 cm	0.5 to 1.0 cm
	log cells $l^{-1}$ sediment	log cells $l^{-1}$ sediment
11.7.78	4.36	4.25
18.7.78	5.02	-

<u>SITE B</u>	Sediment depth	
	Date	
	0.0 to 0.5 cm	0.5 to 1.0 cm
	log cells $l^{-1}$ sediment	log cells $l^{-1}$ sediment
11.7.78	4.31	4.12
18.7.78	4.91	4.42

Standard error of count = 0.107

95% confidence limits of Log MPN are  $\pm 0.213$

Counts are significantly different when  $\log MPN_1 - \log MPN_2$  equals 0.3.

for the much greater nitrification rates observed in these deposits. There seems to be little difference between the population in the top 0.5 cm and those 0.5 to 1.0 cm into the sediment. However, Eh measurements, 0.5 cm into the sediment at this time preclude the occurrence of nitrification. Surface sediments would be prone to mixing by turbulent resuspension or bioturbation, and so could be actively mixed with deeper deposits. Therefore populations may be detected at depths where measured redox potentials indicate they cannot be active.

M.P.N. estimates, of phase I populations, were also attempted on littoral sediments. However, final analysis gave statistically improbable results and therefore even an imprecise estimate of the M.P.N. could not be obtained. Typical results, for site D, are shown below.

Dilution	$2^{-1}$	$2^{-2}$	$2^{-3}$	$2^{-4}$	$2^{-5}$	$2^{-6}$	$2^{-7}$	$2^{-8}$	$2^{-9}$
Number of positive scores	0	0	0	0	2	1	0	1	0

(sampling date 1st August)

If no positive scores were recorded at  $2^{-7}$  dilution it is unlikely that a  $2^{-8}$  dilution would contain nitrifying organisms. Control plates, not receiving an inoculum, showed no presence of nitrite.

Plate 2 shows a typical result for a phase II M.P.N. count from the sediments. Nitrate could not be detected in any of the wells showing a negative test for nitrite. The detection of nitrite at the higher dilutions indicates that nitrogen removal from the systems is a function of the inoculum and not a physical phenomenon (i.e. adsorption). The microbiological mechanism of nitrite removal is not clear (i.e. we cannot determine if it has been oxidised or reduced) and therefore estimates of phase II numbers are impossible.

## 5.7 Methylo-trophic bacteria and nitrification

The discussion concerning the underestimation of phase I population by the M.P.N. counting technique (Section 5.6.2) was based on the assumption that methane oxidising bacteria were not involved in nitrification processes. Also, one possible reason discussed for the  $^{14}\text{C}$ -bicarbonate-N-Serve method underestimating in situ nitrification rates was the participation of methane oxidising bacteria in the oxidation of ammonia to nitrite. This apparent contradiction is reflected in the lack of information of nitrifying activity by methane oxidising organisms in natural environments. It seemed opportune therefore to attempt to investigate this, briefly, in samples taken during the phase of nitrification in the hypolimnion of Grasmere during June and July, 1980.

The method was based on published  $K_m$  values (substrate concentration at half the maximum velocity of reaction) of the methylo-trophic bacteria for the oxidation of methane and ammonia. Dalton (1977) reported these to be  $160\ \mu\text{M}$  for  $\text{CH}_4$  and  $96\ \text{mM}$  for  $\text{NH}_3$  for Methylococcus capsulatus (Bath). If these values are typical then ammonia oxidation should only occur in environments where ammonia concentrations are high and methane is absent (Rudd and Taylor, 1980). Head space analysis of water samples, taken during the nitrification phase of Grasmere, indicated that methane was not present. Addition of methane therefore, should result in decreased rates of nitrate production if the initial oxidation, to nitrite, is mediated by methylo-trophic bacteria. A number of experiments were performed in which the natural ammonia concentration varied between  $20$  and  $60\ \mu\text{g N l}^{-1}$ . Methane was added to give dissolved methane concentrations of between  $200$  and  $500\ \mu\text{g CH}_4\ \text{l}^{-1}$ . This concentration was varied by relating to a calibration curve of volume of pure methane added to dissolved methane concentration, prepared using filter

sterilized lakewater. After methane addition incubation was continued for 5 days. The results were highly variable and, as the number of experiments were few, the data are not presented but will be discussed briefly.

In some cases addition of methane caused a reduction in the rate of nitrate accumulation. However, the magnitude of the reduction appeared to depend on the initial concentration of dissolved methane. The higher methane concentrations completely prevented nitrate accumulation. Kinetic analysis was not possible. In other experiments addition of methane caused no reduction in the rate of nitrate accumulation. The results were therefore inconclusive.

## Chapter 6.

### 6.1 General discussion

There can be little doubt that the change in sewage treatment practice at Grasmere village, and the discharge of the effluent into the River Rothay, have exerted a noticeable effect on the lake. Soluble reactive phosphorus concentrations and areal hypolimnetic oxygen deficits increased significantly ( $P > 0.05$ ) after commission of the sewage works in 1971. Various authorities in the past have considered Grasmere to be a borderline example of a lake between eutrophic and oligotrophic conditions. It now seems probable, based on present estimates of areal oxygen deficits, that it should be classed as eutrophic (Hall et al, 1978). The increased deoxygenation of the hypolimnion of Grasmere was probably caused by the development of higher levels of phytoplankton biomass in the surface waters resulting in greater rates of sedimentation of organic matter into the hypolimnion. Nitrogen and phosphorus are the most common nutrients associated with increased levels of primary production. As phosphorus concentrations in the lake increased by an order of magnitude, and the sewage effluent contributed 98% of the ammonia and 40% of the nitrate concentrations to the main inflow these are the probable causes of increased phytoplankton biomass. Unfortunately chlorophyll a data for the lake were not available before 1971 and therefore comparisons with the more recent data cannot be made. The average summer maximum chlorophyll a concentration, for the years 1971 to 1979 was  $25 \text{ mg m}^{-3}$ , with a range of 10 to  $35 \text{ mg m}^{-3}$  (C.S. Reynolds pers. comm.). The apparent recovery of the lake, in terms of areal oxygen deficits noted between 1974 and 1976 (Hall et al, 1978) was not reflected by changes in chlorophyll a concentrations and therefore may not be due to an improvement in effluent quality. The decreasing areal deficit

between these years could represent a recovery process of the lake, or merely reflect differences in climate. Without further data it is difficult to speculate on this. The lowest seasonal maximum value for chlorophyll a,  $10 \text{ mg m}^{-3}$  was recorded in 1979 and corresponded to the development of extensive macrophyte beds north of the island. Although data are available for only one year it is possible to speculate that these macrophytes were removing nutrients and therefore decreasing the potential for phytoplankton development. The development of macrophytes is significant in the evolution of lakes as they restrict water movements thus increasing the rate of sedimentation and silting. The sequence of nitrogen transformations consisting of ammonification, nitrification and denitrification in the hypolimnion of Grasmere correspond to similar observations in other lakes by Larsen (1976) and Christofi (1978). This sequence is not surprising, in that each stage produces substrate and, in the case of nitrification, suitable conditions for the subsequent process. The demonstration that the nitrification phase was also due to the in situ activity of planktonic chemolithotrophic nitrifying bacteria agrees with the observations of Christofi (1978) on the water column of Blelham Tarn and others on freshwater sediments (Chen et al, 1972; Isirimah et al, 1976). From the increases in nitrate concentration, during the nitrification phase of Grasmere, it can be calculated that this process accounted for between 7 and 16% of the total oxygen consumption in the hypolimnion. These values are larger than those reported by Burns and Ross (1972) of 1.25% for Lake Erie, but much smaller than the 100% noted for certain depth layers in the Lund tubes of Blelham Tarn (Christofi, 1978) and the figures calculated from the nitrate and oxygen concentration data from Lake Mendota (Brezonik, 1968). Calculations based on net increases in nitrate concentration are likely to underestimate the contribution of nitrification to oxygen uptake.

Nitrification was demonstrated in Grasmere when nitrate concentrations were declining. Nitrifying activity in surface sediments may also fail to produce increases in nitrate concentration in the overlying water because of higher levels of nitrate reduction.

The interlake comparisons indicate that a dominant phase of nitrification was a common phenomenon in the lakes of this area which accumulated significant concentrations of ammonia, in the hypolimnion, under aerobic conditions. These lakes also had a hypolimnion whose relative volume was small compared with that of the epilimnion. However, the sustained high nitrate, and low ammonia concentrations observed in Windermere (North basin) suggest that nitrification can occur in lakes which do not share this characteristic. Patterns of nitrification have also been observed in the south basin of Windermere (George, in press). The data from Esthwaite (Heaney, pers. comm.), discussed in Section 5.4.3 indicate that in highly eutrophic lakes chemical changes, characteristic of nitrification, may be compressed on both temporal and depth scales. These observations are in agreement with those on Blelham Tarn (Christofi, 1978), Lake Mendota (Brezonik, 1968) and some Danish lakes (Larsen, 1976). It appears that the occurrence of planktonic nitrification is a common phenomenon in many lakes. In the more eutrophic systems oxygen may become limiting before all the ammonia is utilized. Many earlier workers considered nitrification to be a predominantly benthic process (Mortimer, 1941) and that hypolimnetic deoxygenation was primarily caused by aerobic decomposition processes. It is apparent from the results presented here that planktonic nitrification is important not only in its influence on inorganic nitrogen concentrations within the water column, but also on hypolimnetic deoxygenation. This has been largely overlooked in the past. It should also be noted that increases in hypolimnetic ammonia concentrations are not necessarily due entirely to release from

anaerobic sediments but are also observed in aerobic hypolimnetic water, presumably due to nitrogen mineralization processes.

A main aim of this work was to measure nitrification rates in the hypolimnion of Grasmere, over the period of summer stratification, and to obtain a better estimate of the total contribution of this process to the oxygen deficit. Unfortunately this could not be achieved completely because the  $^{14}\text{C}$  method, as used, underestimated the rate of nitrification. Billen (1976) in describing the method, reported estimates to be within an order of magnitude of the actual rate. Christofi (1978) reported good agreement between results obtained with the method and field nitrate data. No information was presented, in either case, on the possible occurrence of nitrate utilizing processes in situ. Christofi sampled the experimental Lund tubes in Blelham Tarn during an unusual stratification feature in which aerobic water was sandwiched between aerobic layers; it is probable that nitrate transport out of the aerobic system was rapid. Under such circumstances field observations may further underestimate the rate of nitrification if due allowance is not made for the transport of nitrate by turbulence. For example observations of increased concentrations of nitrate in water samples from a particular sampling site, only indicates that nitrification has occurred at some time in the past. In large open systems subject to turbulence increased concentrations of nitrate may not be associated with measurable nitrifying activity at that site but to transport of end products of metabolism from another site. In a confined environment (in a Lund tube) turbulent mixing would be considerably reduced and with a high sampling frequency changes in nitrate concentration would be expected to correlate closely with rates of nitrification. This, however, cannot explain the present data from Grasmere as the nitrification rates were consistently underestimated throughout the whole sampling period.

The maximum measured rate of nitrate production in the water column



of all the lakes in the present study was  $18 \mu\text{g N l}^{-1} \text{ day}^{-1}$ ; this is lower than the maximum measured rate for Blelham Tarn of  $43 \mu\text{g N l}^{-1} \text{ day}^{-1}$  (Christofi, 1978).

The present work on Grasmere was the first attempt to measure nitrification rates in freshwater sediments. The steep Eh gradients found close to the surface of profundal sediments indicated that nitrifying activity was restricted to a top few millimetres near the surface of these deposits. In littoral sediments the Eh discontinuity layer was found deeper in the sediments, possibly reflecting the dissolved oxygen concentration in the overlying water. Eh gradients were not present in littoral sandy sediments possibly due to their porosity to the overlying water or carbon limitation of aerobic heterotrophic metabolism. Profundal sediments might possibly support nitrification to a greater depth during isothermal conditions because of higher oxygen concentrations in the overlying water during winter. Other estimates of nitrifying activity in sediments, using the potential technique, have been made on either the top 5 or 10 cm of deposits (Christofi, 1978; Isirimah et al, 1976) or have sampled to an undefined depth (Chen et al, 1972a). The lakes studied by Christofi and Isirimah et al were shallow and not subject to thermal stratification, the depth to which oxidising conditions persisted was not reported. If these sediments were typical organic rich deposits it is unlikely that oxygen penetrated more than a few cm depth and therefore the experimental conditions may not reflect the situation in situ.

The higher rates of nitrification observed in littoral sediments was in agreement with the findings of Christofi (1978) and Chen et al (1972a) who reported that the nitrification potential of sediments increased with increasing incubation temperatures. Therefore, due to their higher temperatures and greater depths of oxygen penetration, during the summer months, the littoral sediments are more important than profundal sediments

as sites of nitrification. Similar results have been obtained by Jones and Simon (in press). The composition of the sediment is also important as high nitrification rates were not observed in littoral sandy sediments of Grasmere. Billen (1976) on the other hand reported higher rates of nitrification in marine sandy sediments than in muddy deposits. The muddy sediments had steep Eh gradients at 5 to 7 cm depth whilst sediments containing sand either had no Eh gradient or gradients 11 to 14 cm deep. This corresponds to the Eh measurements in this study. The marine sandy sediments studied by Billen contained more nitrate than did the muddy deposits but as no other form of nitrogen was estimated it is impossible to say if it was the dominant inorganic nitrogen species. Niewolak (1970) reported no differences in counts of phase I or II nitrifying bacteria between sandy or muddy freshwater sediments. Nitrification in the sandy deposits of Grasmere could have been limited by low ammonia concentrations ( $< 100 \mu\text{g N l}^{-1}$  interstitial water) although nitrification in the water column continued at substantially lower substrate concentrations than this.

Nitrification rates in oxidised sediments are substantially greater than those estimated for the water column at similar temperatures. Therefore it is likely that the sediments are the most important sites for this process. *Not so when compared on whole lake basis in profundal region.*

In common with those in all other studies the estimated numbers of chemolithotrophic nitrifiers could not account for observed nitrate concentration increases, even though, for the water column at least, these increases were shown to be sensitive to inhibitors of chemolithotrophic nitrification. Such discussions, however, are based on the argument that activities per cell, measured in culture, are comparable to those encountered in the field; this has yet to be proved. In natural situations the activities per cell are likely to be much lower because of nutrient limitation or inhibition. It also appears that culture media

currently in use do not support the growth of all chemolithotrophic nitrifiers present in the inoculum. Despite attempts in the present study to modify the medium, to obtain optimal culture conditions, this problem was still unresolved.

The problems associated with obtaining reliable viable counts of specific bacteria in mixed populations are well established (Buck, 1979). In the case of the chemolithotrophic nitrifiers the nature of the energy source is common to all genera of phase I or II organisms. Clearly the inefficiency of the counting procedure is due to other nutritional deficiencies of the medium.

## 6.2 Suggestions for future research

The within treatment variability of  $\text{CO}_2$  uptake measurements in water, and particularly sediment, samples indicate that the method, although sensitive, lacks precision. This could be improved by increasing the number of replicates per treatment. If these observations are found to be generally applicable to samples from other environments then the results presented by Billen, 1976; Somville, 1978 and Christofi, 1978 must be interpreted with caution since only one sample per treatment was analysed. Other possible inaccuracies of the method include a) the conversion of the N-Serve sensitive  $\text{CO}_2$  uptake to a value for N oxidised, b) the nutritional status of the chemolithotrophic nitrifying organisms in situ and c) the possible participation of the methane oxidising bacteria in nitrification reactions. Therefore the author does not share the enthusiasm of Christofi (1978) and Fenchel and Blackburn (1979), for further use of the method until the above matters are resolved.

It is unlikely that values of the nitrogen oxidised to carbon fixed ratio from cultures can be directly extrapolated to the natural environment because of longer generation times and subsequent greater maintenance energy requirements. It may be possible to obtain more realistic estimates

(of N:C ratios) by adding washed nitrifier cell suspensions, from pure cultures of phase I and II organisms, to natural samples and estimating the ratio under in situ incubation conditions. In the present work N:C ratios of nitrifying cultures, or enrichments, were not determined and the work would have been improved by their inclusion. The demonstration of mixotrophic nutrition in situ would also hamper the attainment of a realistic conversion factor. An investigation of mixotrophic nutrition could involve the addition of radiolabelled organic compounds (acetate, citrate, pyruvate) to samples known to have nitrifying activity. However, these substrates could be oxidised by the heterotrophic microflora and labeled inorganic end products may be assimilated by chemolithotrophic organisms. Fluorescent antibody techniques would also be required to identify the nitrifying bacteria. Techniques for the use of F.A. and autoradiography for these organisms have already been described (Fliermans and Schmidt, 1975).

An alternative approach might involve the simultaneous measurement of N-Serve sensitive oxygen uptake and differences in nitrate concentration changes between inhibited and uninhibited samples. If oxygen uptake, inhibited by N-Serve, was larger than could be accounted for in terms of changes in nitrate concentration this could be interpreted as evidence for the involvement of heterotrophic nutrition of the nitrifying organisms.

Inhibitors of chemolithotrophic nitrifying bacteria which are currently available may be used to distinguish the activity of chemolithotrophic or chemoorganotrophic nitrifiers in samples accumulating nitrate. Of these inhibitors N-Serve has been the most frequently used; its advantages and disadvantages have already been discussed. The sensitivity of the sulphate reducing bacteria to N-Serve only becomes a problem when measuring  $^{14}\text{CO}_2$  uptake, as in the present work. However,

the inhibition of in situ nitrate production by these inhibitors does not preclude the involvement of methane oxidising bacteria in the initial oxidation of ammonia to nitrite. As ammonia oxidising activity seems to be widespread in methylotrophic bacteria it is one area of research into nitrification which is worthy of further study. The use of fluorescent antibodies, produced specifically against the true phase I nitrifying bacteria, would prove useful not only for assessment of the counting efficiency of the various culture techniques, but also for providing information in methylotroph involvement. Low F.A. counts could, however, result from either a small biomass or inactivity of the F.A. towards the ecologically dominant nitrifier species. Since current methods of culture of phase I bacteria are inadequate to distinguish the various ammonia oxidising genera an alternative method for separating the involvement of ammonia or methane oxidisers is required. The method used in the present work, based on published  $K_m$  values of the methylotrophs for  $NH_3$  and  $CH_4$ , was unsuccessful. The basis of the method, is however, worth investigation under more defined conditions. Firstly the headspace in the various sample bottles should be kept constant so that the partition of almost insoluble gases, like methane, between gas and liquid phases is maintained. Analysis, not only for  $CH_4$ , but also of  $CO_2$  concentration should be made to provide information on methane oxidation. A greater range of methane concentrations should be used, possibly with additions of ammonia, to minimise competitive inhibition of the true ammonia oxidisers.

## Summary

1. Observations have been made upon changes in concentration of inorganic nitrogen species in the hypolimnia of lakes Grasmere, Buttermere, Brotherswater, Derwentwater and Windermere.
2. Of the lakes sampled in which ammonia accumulated in aerobic hypolimnetic water a period of nitrification was observed at some time during the period of stratification. The nitrification phase was characterised by increasing nitrate concentrations and decreasing ammonia concentrations. This suggested that nitrification was the dominant nitrogen transformation at that time.
3. In Grasmere the nitrification phase occurred over a 4 to 5 week period in June and July and was found to be due to the activity of planktonic bacteria which were sensitive to inhibitors of the chemolithotrophic nitrification process. Using the observed nitrate increases, and stoichiometric equations, it was calculated that chemolithotrophic nitrification accounted for 7 to 15% of the total oxygen consumption of the hypolimnion.
4. Using the  $^{14}\text{C}$ -bicarbonate and N-Serve inhibition method of Billen (1976) attempts were made to estimate rates of nitrification in the hypolimnion of Grasmere. Two peaks of activity were found in May and July. Only the latter coincided with a nitrification phase as defined above. These observations were confirmed in other lakes. Therefore oxidation of ammonia to nitrate could occur but remain undetected at times when nitrate uptake was the dominant process. This is particularly true of sediments and therefore any calculations of oxygen demand based solely on increases in nitrate concentration will be underestimates.

5. Comparison of the rates of change in field nitrate concentrations and the cumulative nitrate concentration (calculated from the estimated nitrification rates) indicated that the  $^{14}\text{C}$ -bicarbonate and N-Serve inhibition method underestimated the actual rate of nitrification. Possible reasons for this are discussed.
6. Nitrification in the sediments of Grasmere was also investigated. Oxidising conditions, and therefore nitrification, were restricted in profundal sediments to the top few millimetres of the deposits. In littoral sediments the oxidised layers penetrated deeper into the deposits. This may have been related to oxygen concentrations in the overlying water although the physical characteristics of sediments were also important.
7. At comparable temperatures rates of nitrification in the sediments were two orders of magnitude greater than those estimated in the water column. Because of higher temperatures in this zone littoral sediments showed greater nitrifying activity than profundal sediments. Within the littoral zone the physical characteristics of the sediment were also important in determining the nitrification rate. Consistently lower rates were found in sandy sediments than in organic rich deposits.
8. Nitrate was the dominant inorganic nitrogen species in sandy sediments whereas ammonia was dominant in all organic rich deposits. This was probably due to the limitation of nitrate reduction by oxygen, or organic carbon, in sandy sediments.
9. In the organic rich sediments of Grasmere published values of nitrate reductase activity were two orders of magnitude greater than the maximum estimated nitrification rate. This indicates that nitrate reduction is the primary factor in determining the nitrate concentration in the interstitial waters of these sediments.

10. Only enrichments in dilute basal medium with low substrate concentrations resulted in the reliable detection of ammonia oxidising activity within reproducibly short incubation times. Under these conditions M.P.N. counts of phase I nitrifiers showed a population maximum of 17 bacteria ml<sup>-1</sup> during the nitrification phase in the hypolimnion of Grasmere. Rates of ammonia oxidation, obtained with cultures, indicated that the M.P.N. values obtained could not account for the observed increases in nitrate concentration.

11. Comparison of M.P.N. counts from oxidised sediment and water samples, at similar in situ temperatures, indicated that sediments have significantly greater ( $P < 0.05$ ) populations of phase I nitrifying bacteria.



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