1 Introduction

Nitrogen is, without question, one of the most important elements required by biological systems and in many aquatic ecosystems can be considered a factor limiting primary productivity. It is, therefore, of prime importance to establish the mechanisms by which nitrogen may be cycled in aquatic environments, and to obtain quantitative data on those microorganisms that perform many of the transformations which make up the nitrogen cycle. Aspects of the nitrogen cycle in aquatic environments have been the subject of recent reviews (for example, see Painter, 1970; Keeney, 1972). In the present review, we shall be concerned only with those processes in which inorganic nitrogen is
converted to organic nitrogen, and the microorganisms responsible for such assimilatory reactions. We will consider, therefore, aspects of the reduction of atmospheric dinitrogen \((\text{N}_2)\) and nitrate \((\text{NO}_3^-)\) to ammonia \((\text{NH}_3)\), and the incorporation of ammonia into organic compounds; such considerations will relate particularly, whenever possible, to those organisms of significance in aquatic environments.

Data on the levels of fixed inorganic sources of nitrogen in aquatic ecosystems are scattered throughout the literature, and, due to the many transformations undergone by such compounds, their concentrations vary over a wide range both on a seasonal basis and even within a 24-hour period as a result of the mixing of different bodies of water and the activities of phyto- and zooplankton. Vollenweider (1968), quoted by Keeney (1972), has provided a list of inorganic nitrogen concentrations in freshwater lakes of different trophic levels. On this basis, an oligotrophic environment contains less than \(1 \times 10^{-6} \text{ M}\) nitrogen and a eutrophic environment contains a concentration of nitrogen greater than \(1.4 \times 10^{-6} \text{ M}\). These figures agreed well with the data of Lueschow \textit{et al.} (1970), again quoted by Keeney (1972), on the nitrogen levels of lakes in Wisconsin. Oligotrophic Lake Crystal showed a monthly mean nitrogen content of \(0.6 \times 10^{-6} \text{ M}\) in a range of \(0.3-0.9 \times 10^{-6} \text{ M}\), whilst eutrophic Lake Mendota showed a monthly mean of \(2.9 \times 10^{-6} \text{ M}\) in a range of \(1.0-5.1 \times 10^{-6} \text{ M}\). Inorganic nitrogen levels in the sea, in general, appear to correspond to those of oligotrophic freshwater environments; for example, early results quoted by Sverdrup \textit{et al.} (1946) give nitrate levels corresponding to the range of \(0.07-3.0 \times 10^{-6} \text{ M}\) nitrogen, nitrite \(0.007-0.25 \times 10^{-6} \text{ M}\) nitrogen and ammonia \(0.03-0.25 \times 10^{-6} \text{ M}\) nitrogen. It should be emphasized, however, that nitrate is often found at a subsurface maximum at a depth of several hundred metres, with much lower values in the euphotic zone. A region of particularly high surface concentrations of inorganic nitrogen is the Peru Current, where Wooster \textit{et al.} (1965) reported nitrate levels corresponding to \(1.4-2.1 \times 10^{-6} \text{ M}\) nitrogen and Eppley \textit{et al.} (1970) reported nitrate levels, at all stations sampled, exceeding \(1.1 \times 10^{-6} \text{ M}\) nitrogen. In the English Channel (Cooper, 1933) the surface nitrate level was about \(0.6 \times 10^{-6} \text{ M}\) in winter and early spring, but fell later in the year, presumably due to phytoplankton growth. Lower values have been recorded in the Sargasso Sea off Bermuda (Riley, 1957; Ryther \textit{et al.}, 1961) and in Sagami Bay off Japan (Miyazaki \textit{et al.}, 1973). The nitrite level in the sea is usually very
low in surface waters (Ryther et al., 1961; Hattori and Wada, 1971; Miyazaki et al., 1973), but appreciable concentrations, in the order of \(0.2 \times 10^{-6}\) M nitrogen, are often found at a subsurface maximum in oxygen-depleted waters (see Wooster et al., 1965), and is thought to be largely produced by the action of denitrifying bacteria (Brandhorst, 1959, quoted by Wooster et al., 1965; Thomas, 1966; Fiadeiro and Strickland, 1968; Carlucci and Schubert, 1969). Ammonia may also be an important nitrogen source in freshwater and marine environments (Dugdale and Goering, 1967; Keeney, 1972). In the English Channel the ammonia level was reported as \(0.2 \times 10^{-6}\) M nitrogen, but this level may rise to \(0.9 \times 10^{-6}\) M nitrogen in coastal waters (Cooper, 1933), while Eppley et al. (1969a) have reported values of up to \(1.0 \times 10^{-6}\) M nitrogen in the Pacific Ocean. Ammonia concentrations appear to be variable, probably due to a very rapid turnover in the planktonic population (Goering et al., 1964; Beers and Kelly, 1965; Eppley et al., 1971). Another nitrogen source of possible significance in aquatic environments is urea, derived either as an excretory product of zooplankton and higher animals or from pollution. Urea may serve as sole nitrogen source for many eukaryotic algae (Naylor, 1970), blue-green algae (Fogg et al., 1973), and natural marine phytoplankton populations (McCarthy and Eppley, 1972). The breakdown of urea to ammonia has been studied in eukaryotic algae by Leftley and Syrett (1973). These authors showed that two routes may be operative, namely urease and ATP urea amidolyase; members of the Chlorophyceae contained the ATP-dependent system but not urease, while in the other organisms studied, including Tetraselmis, Monochrysis and Phaeodactylum, the reverse was true.

Another important inorganic source of nitrogen of particular significance to aquatic habitats is dinitrogen (atmospheric nitrogen) and most attention has been given, in this context, to fixation by blue-green algae (see below). From the data of Murray et al. (1969), the saturation concentrations of dissolved dinitrogen may be calculated to range from \(16.4 \times 10^{-4}\) M nitrogen in distilled water at 0 °C to \(7.8 \times 10^{-4}\) M nitrogen in water of chlorinity 20 per cent at 25 °C; thus concentrations in aquatic environments will vary within these limits with largest fluctuations being expected in estuarine situations. Therefore, the concentration of nitrogen due to dissolved dinitrogen is substantially greater than the concentration of nitrogen due to the other inorganic, fixed nitrogen compounds discussed above.
In a survey of lakes in southern Wisconsin, Gerloff and Skoog (1957) concluded that nitrogen was most likely to be the factor limiting algal growth. Skelef et al. (1971) further suggested that nitrogen may be the common algal growth-limiting factor in those lakes in which phosphorus is relatively abundant, and also in waters polluted with domestic waste. Thomas (1970) studied phytoplankton populations in the nutrient-deficient waters of the tropical Pacific Ocean, in which nitrate was not detectable in surface waters and ammonia was present at less than \(0.07 \times 10^{-6}\) M and applied data from the kinetics of ammonia uptake to calculate the population growth rate. These results agreed well with the rates calculated from \(^{14}\text{C}\) productivity and chlorophyll concentrations and thus indicated that growth of the population was limited by the availability of ammonia nitrogen. Further evidence that nitrogen may be the limiting nutrient in the open sea, in coastal and estuarine environments has been cited by Ryther and Dunstan (1971), Thomas and Owen (1971), Caperon and Meyer (1972a), Morris et al. (1972), Goldman et al. (1973) and Thayer (1974).

The available evidence indicates, therefore, that the concentrations of fixed nitrogen sources in the majority of aquatic environments are low, and that although such nitrogen may not always be the primary limiting nutrient, it is potentially limiting under most conditions. Nitrogen fixation, however, is unlikely to be limited by dinitrogen availability and other possible constraints of this system are discussed below.

2 Mechanisms

As outlined above, the principal inorganic sources of nitrogen available to aquatic microorganisms are dinitrogen \((\text{N}_2)\), nitrate \((\text{NO}_3^-)\) and ammonia. The term "ammonia" is used throughout the present review to denote the substrate, whether the form in which it is taken into the cell and subsequently metabolized be ammonia \((\text{NH}_3)\) or ammonium ion \((\text{NH}_4^+)\). The \(pK\) of the reaction

\[
\text{NH}_4^+ \leftrightarrow \text{NH}_3 + \text{H}^+ 
\]

is about 9.2 (Brown et al., 1974), so that the predominant species in most aquatic environments would be ammonium, as would be the case in the majority of cells since intracellular pH values usually fall below this figure. Ammonia, in addition to being a possible source of nitrogen, plays a crucial intermediate role in the assimilatory reduction of
dinitrogen and nitrate. These interrelationships are shown in Fig. 1. The mechanisms involved in the assimilatory reduction of dinitrogen, nitrate and nitrite, resulting in the formation of ammonia, will be discussed first and will be followed by an account of the integration of ammonia (either as primary source of nitrogen or as intermediate) into cellular metabolism.

Fig. 1. Principal routes of assimilation of inorganic nitrogen in microorganisms. a, Bacteria; b, blue-green algae; c, eukaryotic algae; d, fungi.

Whilst dissimilatory processes are beyond the scope of the present review, the vital role played by microorganisms in the overall cycling of nitrogen in aquatic environments should not be overlooked, and in this context the reader is referred to the review of Painter (1970).

2.1 DINITROGEN FIXATION

The ability to reduce dinitrogen to ammonia, i.e. nitrogen fixation, is of vital ecological importance, and appears, at present, to be a property restricted to the prokaryotic microorganisms (bacteria and blue-green algae). The distribution of the ability to fix nitrogen amongst such organisms has been considered recently by Postgate (1971), Benemann and Valentine (1972), and Stewart (1973).

Of particular importance to marine, brackish, and freshwater environments are the free-living organisms, and in such habitats many bacteria have been found to be able to fix nitrogen, although to differing degrees. Members of the three photosynthetic families, Thiorhodaceae, Athiorhodaceae and Chlorobacteriaceae, have been
shown to be nitrogen fixers, and these organisms are important in the quantitative sense in view of their potential independence of exogenous carbon sources. Of the free-living heterotrophic bacteria found in aquatic environments those able to fix nitrogen include members of the family Azotobacteriaceae, and members of the genera *Clostridium*, *Bacillus*, *Mycobacterium* and *Desulphovibrio*.

Blue-green algae are ubiquitous in freshwater, marine and estuarine environments, being quantitatively possibly the most important group of nitrogen-fixing organisms. Their widespread distribution is probably largely due, as in the case of photosynthetic bacteria, to their possible independence of organic carbon sources. Those members of the filamentous heterocystous group tested are virtually all able to fix nitrogen, whereas the ability appears to be more restricted within the filamentous, nonheterocystous group (Fogg *et al.*, 1973). Similarly, few unicellular strains have been shown to fix nitrogen, the first genus to be implicated being *Gloeocapsa* (Wyatt and Silvey, 1969).

The reduction of dinitrogen to ammonia involves a six electron change, and is believed to take place in the following three two-electron steps:

\[
\begin{align*}
2e^- & \rightarrow 2e^- \rightarrow 2e^- \\
N = N & \rightarrow HN = NH \rightarrow H_2N = NH_2 \rightarrow 2NH_3 \\
2H^+ & \rightarrow 2H^+ \rightarrow 2H^+
\end{align*}
\]

Dinitrogen  Diimide  Hydrazide  Ammonia

However, intermediates have never been isolated (see Dalton and Mortenson, 1972), which probably reflects the tightly coupled nature of the process. The enzyme complex responsible for the fixation of dinitrogen is termed “nitrogenase”. This has been resolved and is well characterized in the case of bacteria (see Postgate, 1971; Benemann and Valentine, 1972; Brown *et al.*, 1974), but studies with blue-green algae are rather less advanced (see Fogg *et al.*, 1973; Brown *et al.*, 1974). The majority of studies have indicated blue-green algal nitrogenase to be soluble, but Gallon *et al.* (1972) have reported that of *Gloeocapsa* to be sedimented at 10000 g.

It would appear that in all nitrogenases so far examined, two major protein components are present, and that both are necessary for nitrogen fixation to take place. The larger protein contains both molybdenum and nonhaem iron, whilst the smaller component contains only nonhaem iron. Some degree of complementarity of nitrogenase
proteins isolated from different organisms is observed, particularly if
the organisms are closely related physiologically. The smaller of the
two nitrogenase components is extremely oxygen sensitive, and this
renders the overall process oxygen sensitive. This presents no problem
to anaerobic organisms, but aerobes are faced with the difficulty of
preventing oxygen reaching the site of nitrogen fixation. Aerobic
bacteria, e.g. Azotobacter sp., are thought to achieve this by respiratory
and/or conformational protection (Postgate, 1971). The situation with
respect to oxygen protection in blue-green algae is not clear. It was
first proposed by Fogg (1949) that in heterocystous algae, nitrogen
fixation is largely confined to the heterocysts, which have subsequently
been shown to provide a localized anaerobic environment, and now
there is a good deal of evidence in support of this proposition (Kulasoorija et al., 1972; Fogg et al., 1973; Carr and Bradley, 1973; Fay, 1973;
Weare and Benemann, 1973). However, several workers have contested
this view showing nitrogenase activity to be equally distributed between
heterocysts and vegetative cells (Smith and Evans, 1970, 1971; Ohmori
and Hattori, 1971). In situ studies on nitrogen fixation, however, tend
to support the former case (Horne and Goldman, 1972; Horne et al.,
1972), while Thomas and David (1972) have presented evidence that
suggests that nitrogenase activity is maximal in two- to four-day-old
heterocysts and decreases markedly as these cells age. There is also
evidence (summarized by Fogg et al., 1973) to suggest that in non-
heterocystous filamentous algae nitrogen fixation takes place only
under microaerophilic conditions. Wyatt and Silvey (1969) have
claimed that nitrogen fixation rates in the unicellular alga Gloeocapsa
minor, grown aerobically, are comparable with heterocystous algae,
whilst Wyatt and Stewart (cited by Stewart, 1971) have demonstrated
higher activities of nitrogenase in Gloeocapsa grown microaerophilically
than grown aerobically. Thus whilst nitrogenase appears to be present
in vegetative cells of blue-green algae (at least under microaerophilic
conditions), it is argued that the presence of the enzyme in heterocysts
appears to be necessary for nitrogen fixation under aerobic conditions.

Two prerequisites for nitrogen fixation are:

a. a source of reducing power; and
b. a source of energy (although this latter requirement is not fully
understood since the conversion of dinitrogen to ammonia could,
in theory, be exergonic (see Postgate, 1971)).
In photosynthetic organisms photoreduction and photophosphorylation could supply the reducing power and energy required for nitrogen fixation, and this may partially explain the quantitative contribution of such organisms to total nitrogen fixation in aquatic environments. The possible sources of reducing power and energy for nitrogen fixation in both photosynthetic and nonphotosynthetic organisms have been described recently by Benemann and Valentine (1972) and Brown et al. (1974) and the reader is referred to these articles for further discussion.

2.2 NITRATE REDUCTION

Throughout this review the term "nitrate reduction" refers to the assimilatory process.

As discussed above (see section 1) nitrate is probably the most important inorganic source of fixed nitrogen in aquatic habitats, particularly in subsurface layers. The capacity to utilize such nitrate as sole nitrogen source is a property widespread in microorganisms. The majority of bacteria and blue-green algae isolated from waters have been shown to assimilate nitrate, and this appears to be a common property of eukaryotic microalgae. However in yeasts (van der Walt, 1971) and other microfungi nitrate utilization appears to be a more restricted property.

The reduction of nitrate to ammonia requires at least two enzyme systems, namely nitrate reductase and nitrite reductase, which catalyse the following sequence of reactions:

\[
\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \rightarrow \rightarrow \text{NH}_3
\]

Nitrate Nitrite Ammonia

A variety of intermediates between nitrite and ammonia have been proposed, and these, together with evidence for their existence, have been discussed by Painter (1970). The reader is also referred to the review of Payne (1973) for a general discussion of the reduction of nitrogenous oxides by microorganisms.

2.2.1 Nitrate reductase

Information concerning bacterial nitrate reductase is rather scanty (Nason, 1962; Takahashi et al., 1963; Hewitt and Nicholas, 1964). Nicholas and Nason (1955) purified a soluble enzyme from \textit{E. coli B},
which was NAD linked, and recently Guerrero et al. (1973) have isolated and characterized a soluble enzyme from *Azotobacter chroococcum*. In the latter case, the physiological electron donor remains a mystery since NADH, NADPH, FADH$_2$ and FMNH$_2$ were all rather ineffectual. On the basis of tungstate inhibition of the enzyme, the authors suggest that molybdenum may be important to activity. In a marine psychrophilic strain of *Pseudomonas* (D. S. Macdonald-Brown and C. M. Brown, unpublished), the assimilatory nitrate reductase was soluble (after 30 min at 100000 x g), required NADH and FAD for activity and was rather unstable, activity being lost upon dialysis.

Blue-green algal nitrate reductase is a molybdo-protein complex (see Fogg et al., 1973). The enzyme isolated from *Anabaena cylindrica* is particulate (Hattori and Myers, 1967), but can be solubilized by sonication in the presence of the detergent Triton X-100 (Hattori, 1970). Nitrate reduction is stimulated in the light, and thus photo-reduction (via ferredoxin) may be the physiological source of electrons. However, both NADH and NADPH have been shown to stimulate nitrate reductase activity in cell-free extracts (see Wolk, 1973).

The situation with respect to nitrate reductase in fungi is a little clearer. Preliminary experiments with the yeasts *Hansenula anomala* (Silver, 1957; Pichinoty and Méténier, 1966) and *Candida utilis* (Sims et al., 1968) indicated the enzyme to be a metalloflavoprotein requiring NADH or NADPH for activity. Recently Rivas et al. (1973) have isolated a soluble enzyme from *Torulopsis nitratophila* which required FAD and NADH or NADPH for activity while Burn et al. (1974) have shown the enzyme from *Candida utilis* to be soluble, NAD(P) linked, and stimulated by the presence of FMN and molybdenum. In general, the nitrate reductases of the filamentous fungi thus far examined are soluble molybdo-flavoproteins requiring reduced nicotinamide nucleotides as electron donors, and in some cases may be cytochrome linked (see Brown et al., 1974).

Nitrate reduction in eukaryotic algae (and higher plants) is catalysed by a NAD-linked molybdo-flavoprotein, an enzyme complex of high molecular weight (Vega et al., 1971; Relimpio et al., 1971; Hageman and Hucklesby, 1971). Solomonson and Vennesland (1972) have examined the nitrate reductase from the unicellular alga *Chlorella vulgaris* and found it to be associated with a type b cytochrome. The enzyme from *Dunaliella tertiolecta* has been isolated by LeClaire and Grant (1972), who reported it to be a high molecular weight (500000...
daltons) molybdo-flavoprotein which may be either NAD or NADP linked. As with the prokaryotic algae, light has been reported to stimulate nitrate reduction in several eukaryotic algae (Grant and Turner, 1969), although it is unknown to what extent photoreduction may be involved in the process. Epplcy and Coatsworth (1968) who demonstrated light-induced nitrate reduction in *Ditylum brightwellii* have suggested that two reductive pathways – (a) NAD-linked, (b) linked directly to photosynthesis – may be operative, and NAD-linked nitrate reductase has been demonstrated in a variety of marine phytoplankters (Epplcy *et al.*, 1969a). The light dependence of the nitrate reductase of marine phytoplankton has been further investigated by MacIsaac and Dugdale (1972) and Packard (1973). The latter worker has shown that such light dependence shows a rectangular hyperbola, and $K_L$ values of 0.002–0.03  langley were obtained, indicating that the light requirements of phytoplankton are satisfied even at low light intensities.

### 2.2.2 Nitrite reductase

As was the case with nitrate reductase, information concerning bacterial assimilatory nitrite reductase is also rather sparse (Nason, 1962; Takahashi *et al.*, 1963; Hewitt and Nicholas, 1964). Spencer *et al.* (1957) discovered a soluble enzyme in *Azotobacter vinelandii* that reduced nitrite and hydroxylamine, and which utilized reduced nicotinamide nucleotides as electron donors and required flavin nucleotides for maximum activity. Cole (1968) listed three distinct nitrite reductase activities in *E. coli*, but only one of these (NAD-linked) appears to be responsible for physiological nitrite reduction (Kemp and Atkinson, 1966). Prakash *et al.* (1966, 1972) have obtained from nitrate grown *Achromobacter fisheri* a haemoprotein which will catalyse the reduction of nitrite and hydroxylamine to ammonia, but its physiological significance remains doubtful. Recently Vega *et al.* (1973) have prepared a soluble nitrite reductase from nitrate grown *Azotobacter chroococcum*. This is an NAD-linked FAD-dependent metalloprotein, which in contrast with nitrate reductase does not appear to contain molybdenum.

Nitrate reductase also appears to be a soluble enzyme in the blue-green alga *Anabaena cylindrica* (Hattori and Myers, 1966, 1967), and has been partially purified (Hattori and Uesugi, 1968a, b) and separated from hydroxylamine reductase activity (Hattori and Uesugi, 1968a). Electron donors for the enzyme may be photoreduced ferredoxin
or NADPH (unlike the bacterial system) in the presence of a diaphorase.

Rivas et al. (1973) have demonstrated a soluble NADP-linked FAD-dependent nitrite reductase in the yeast *Torulopsis nitratophila*, and suggest that the enzyme consists of two moieties, a FAD-dependent NADPH diaphorase and a terminal nitrite reductase metalloprotein. A similar picture is seen in filamentous fungi; Nason et al. (1954) partially purified from *Neurospora crassa* a nicotinamide nucleotide-linked FAD-dependent nitrite reductase, which was a metalloprotein. The enzyme was further purified by Nicholas et al. (1960) who concluded that it was an NAD-linked FAD-dependent protein containing iron, copper and thiol groups.

The enzyme from those eukaryotic algae examined shows similarities to that isolated from blue-green algae. Zumft (1972) has shown nitrite reductase from *Chlorella* to be a soluble enzyme which is electrophoretically separable into two components, each of which is able to reduce nitrite and hydroxylamine. It is a ferredoxin-linked haemoprotein (2 Fe per mole), but little evidence is available as to the influence of light on the enzyme, and so the role of photoreduced ferredoxin as physiological electron donor remains open. A good deal of work upon the nitrite reductase of marine phytoplankton has been reported (Grant, 1967; Eppley et al., 1969a, b; Eppley and Rogers, 1970; Lui and Roels, 1972), and the enzyme appears to be stimulated by light, suggesting that photoreduction may be important. Grant (1970) has isolated and purified soluble nitrite reductase from *Dunaliella tertiolecta*; the enzyme is ferredoxin linked and will not accept electrons from NADH or NADPH even in the presence of a diaphorase.

### 2.3 AMMONIA ASSIMILATION

The importance of ammonia to microbial nitrogen metabolism is two-fold. First, in some natural ecosystems it may provide a sole source of nitrogen, and second, it plays an intermediate role in the utilization of dinitrogen, nitrate and nitrite. It is hardly surprising, therefore, that the ability to utilize ammonia is ubiquitous among microorganisms, although the pathways involved may be rather variable. In most aquatic habitats, ammonia is unlikely to be the principal source of nitrogen (except under pollution conditions where the concentration of ammonia may be high due to deamination of nitrogenous organic
compounds), and thus so far as most aquatic microorganisms are concerned, ammonia is of greatest importance as an intermediate in the utilization of other compounds.

There appear, at the present time, to be two major routes of ammonia incorporation in bacteria, namely the amino acid dehydrogenases and the glutamine synthetase/glutamate synthase (GOGAT) couple. These pathways will be outlined here and for further discussion the reader is referred to Brown et al. (1974). Amino acid dehydrogenases are soluble enzymes that catalyse the reductive amination of 2-oxo acids by ammonia to yield the corresponding two amino acids. The presence of glutamic dehydrogenases (GDH) is widespread in bacteria; such enzymes may be either NADP or NAD linked, the former type being implicated in biosynthesis, the latter in degradation. Although NADP-linked GDH is thought to be biosynthetic, its relatively high $K_M$ for ammonia (usually in the region of 10–25 mM) casts doubt upon its efficiency of functioning at low physiological levels of ammonia; consequently it is believed that the enzyme is only quantitatively important when ammonia concentrations are high. NADP-linked alanine dehydrogenase has been reported to be present in Bacillus, Mycobacterium, and Streptomyces species (see Brown et al., 1974), but Meers and Kjaergaard Pedersen (1972) have concluded that on the basis of (a) a high $K_M$ value for ammonia (in Bacillus licheniformis it was found to be 300 mM), and (b) that maximal activity was found in cells grown in the presence of alanine, this enzyme in vivo is more likely to fulfil a catabolic role. Similarly, other amino acid dehydrogenases (Sanwal and Zink, 1961; Poralla, 1971) have been allocated a principally catabolic function. Bacterial glutamine synthetase is a soluble, polymeric enzyme, which may contain divalent cations, catalysing the ATP-dependent amination of glutamate to glutamine, and which is subject to an array of control mechanisms (see Shapiro and Stadtman, 1970). Although this enzyme has a low $K_M$ for ammonia ($< 1.0$ mM), its quantitative significance does not lie in the synthesis of glutamine per se, but rather in coupled reactions (see below) with glutamine as intermediate. Carbamyl phosphate is an intermediate in the synthesis of arginine and pyrimidines, and its synthesis is catalysed (in microorganisms) by the glutamine-dependent carbamyl phosphate synthetase:

$$\text{L-Glutamine} + 2\text{ATP} + \text{HCO}_3^- + \text{H}_2\text{O} \rightarrow \text{Carbamyl phosphate} + 2\text{ADP} + \text{P}_i + \text{L-Glutamate}$$

Thus the glutamine synthetase/carbamyl phosphate synthetase couple
may also have had a part to play in the assimilation of ammonia, although probably not in the quantitative sense. It should be pointed out that ammonia itself may act as alternative substrate to glutamine, but its high $K_M$ ($\sim 100$ mM) makes its significance as a primary uptake mechanism rather doubtful.

Tempest et al. (1970) first discovered in *Aerobacter aerogenes* an enzyme which was named glutamine (amide): 2 oxoglutarate amino transferase (oxido reductase NADP)—GOGAT. It catalyses the following reaction:

$$\text{Glutamine} + 2 \text{ oxoglutarate} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{ Glutamate} + \text{NADP}$$

This enzyme has since been given the trivial name glutamate synthase (Prusiner et al., 1972) and has been found in a variety of bacteria including isolates from marine, estuarine and freshwater locations (see Brown et al., 1974). It may be NADP or NAD linked, is soluble, and in one case, namely *E. coli*, its structure has been determined (Miller and Stadtman, 1973). Thus this enzyme, in conjunction with glutamine synthetase, provides an alternative route for the net synthesis of glutamate (and hence other amino acids and nitrogenous compounds) from ammonia. There is good evidence to suggest that this pathway may be especially important at low concentrations of ammonia (see Brown et al., 1974), and this may well be of particular relevance in aquatic habitats. The metabolic disadvantage of the pathway is the necessary expenditure of 1 mol ATP mol$^{-1}$ of ammonia assimilated, but this may represent the price to be paid for the ability to "scavenge" ammonia. Such a scavenging role could be significant in nitrogen-fixing organisms and, indeed, glutamate synthase does appear to be present in a variety of nitrogen fixing bacteria (Nagatani et al., 1971; Dainty, 1972; Drozd et al., 1972).

The situation with respect to ammonia assimilation in blue-green algae is less clear. Both NADP- and NAD-linked glutamic dehydrogenases have been detected in a variety of organisms (Pearce et al., 1969; Scott and Fay, 1972; Neilson and Doudoroff, 1973; Haystead et al., 1973; Batt and Brown, 1974) but the activities reported are very low. Similarly NAD-linked alanine dehydrogenase seems to be widespread (Neilson and Doudoroff, 1973; Stewart, 1973; Haystead et al., 1973; Batt and Brown, 1974) although with low activity — and extremely low levels of NADP-linked alanine dehydrogenase have been reported in several organisms (Scott and Fay, 1972; Neilson and Doudoroff,
The low activities observed, coupled with apparent $K_M$ values for ammonia of 10 mM for GDH and 5 mM for ADH (Haystead et al., 1973), make the roles of these enzymes in ammonia assimilation (especially at low concentrations) rather uncertain. Glutamine synthetase activity has been detected in cultures of *Anabaena cylindrica*, *Anaebana flos-aquae* and *Westiellopsis prolifica* (Dharmawardene et al., 1972, 1973; Haystead et al., 1973; Batt and Brown, 1974), and it is believed on the basis of low $K_M$ values for ammonia (about 1 mM) that this enzyme could be responsible for the assimilation of ammonia produced by nitrogen fixation. In this respect Dharmawardene et al. (1973) found that on a protein basis heterocysts contain nearly twice as much of the enzyme as do the vegetative cells. They also detected very low levels of NADP-linked glutamate synthase in extracts of *Anabaena cylindrica* (Dharmawardene et al., 1972), although Neilson and Doudoroff (1973), and Batt and Brown (1974) were unable to detect this enzyme in any of the blue-green algae that they tested. The activities of carbamyl phosphate synthetase and various other enzymes possibly involved in ammonia assimilation have been examined in *Anabaena cylindrica* by Haystead et al. (1973) and Batt and Brown (1974) but the results appear to be rather inconclusive.

Until recently, the only accepted pathway of ammonia assimilation in yeasts and other fungi was the synthesis of glutamic acid via glutamic dehydrogenase. An NADP-linked enzyme is present in the majority of yeasts, and under conditions of ammonia limitation large quantities of the enzyme are synthesized (see Brown et al., 1974). This has been suggested by Brown and Stanley (1972) to be a possible mechanism by which yeasts can efficiently assimilate ammonia, since the $K_M$ of glutamic dehydrogenase for this substrate is, as in bacteria, rather high. Glutamine synthetase activity is usually detectable in most yeasts (depending upon the cultural conditions) and this may play a part in assimilation. The role of glutamine synthetase in yeasts has been recently investigated by Sims and Ferguson (1974) and Ferguson and Sims (1974a, b). Brown et al. (1973a) have reported the presence of a NAD-linked glutamate synthase in the fission yeasts *Schizosaccharomyces pombe* and *Schizosaccharomyces malidevorans*. Johnson and Brown (1974) have extended this study in a systematic examination of a variety of yeasts, and found the enzyme also to be present in *Saccharomycodes ludwigii*. Thus, it is possible that the GS/GOGAT couple may be responsible for ammonia assimilation in both prokaryotic and eukaryo-
tic organisms, although this pathway, at the present time, appears to be more widespread in prokaryotes. Glutamine-linked carbamyl phosphate synthetase is also found in yeasts and must also play a role in ammonia assimilation. An NAD-linked glutamic dehydrogenase has been detected in the unicellular water mould *Blastocladiella emersonii* (Le John and Jackson, 1968), whilst in mycelial fungi “biosynthetic” NADP-linked glutamic dehydrogenase is predominant. Glutamate synthase has not been detected in two strains of *Neurospora* and *Aspergillus* that have been examined (B. Johnson, unpublished observations). As in yeasts, glutamine synthetase and carbamyl phosphate synthetase are found in more complex fungi, and must play an important role, albeit quantitatively minor, in ammonia assimilation.

Although data are rather sparse, glutamic dehydrogenase also appears to be the enzyme principally responsible for ammonia assimilation in eukaryotic algae. Morris and Syrett (1965) detected NADPH-linked glutamic dehydrogenase activity in both nitrate and ammonia grown cultures of *Chlorella vulgaris*, and found the enzyme to be soluble and constitutive. However, Talley *et al.* (1972) have demonstrated the presence of both NAD- and NADP-linked glutamic dehydrogenases in a thermophilic strain of *Chlorella pyrenoidosa*, and further have shown the NADP-linked enzyme to be inducible by ammonia. NADP-linked glutamic dehydrogenase activity has been demonstrated by Eppley and Rogers (1970) in *Ditylum brightwellii*, and in common with the glutamic dehydrogenases isolated from other sources, this enzyme showed a fairly high $K_M$ for ammonia (10 mM) which, unless the organism concentrates ammonia intracellularly, makes the significance of the enzyme doubtful at low ammonia concentrations such as prevail in the majority of aquatic environments. McCarthy and Eppley (1972) detected both NADP- and NAD-linked GDH in mixed cultures of marine phytoplankton, whilst Eppley *et al.* (1971) found only the NAD-linked enzyme to be present in *Coccolithus huxleyi*.

It can be seen, in retrospect, that a variety of mechanisms exist which allow aquatic microorganisms to assimilate efficiently the generally low concentrations of nitrogen available in their environments. The relative efficiency of such mechanisms in different organisms is of vital importance so far as competition for nitrogen is concerned, and in this respect the possible influence of the environment upon the physiology of nitrogen assimilation in natural populations cannot be ignored. Such considerations are outlined in section 3.
3 Physiological and ecological aspects

3.1 BACTERIA

3.1.1 Nitrogen fixation

The contribution of free living bacteria to nitrogen fixation in aquatic environments is unclear, although nitrogen-fixing organisms such as *Azotobacter*, *Clostridium*, *Desulphovibrio* and photosynthetic bacteria may be isolated from the water column, from the surface of macrophytes and from sediments (see Stewart, 1971, and Keirn and Brezonik, 1971). Pshenin (1963) carried out a systematic survey of the distribution of *Azotobacter* and other nitrogen-fixing bacteria in the Black Sea together with observations on the distribution of phytoplankton. In the water column the numbers of *Azotobacter* were higher in summer than in winter and there was a direct relationship between the bacterial number and that of the "large forms" of phytoplankton. It was proposed that, in sea water, the carbon source of *Azotobacter* was the moribund cells of these large forms of phytoplankton together with other vegetable detritus (which was more abundant in summer). *Azotobacter* was found in greater numbers on the surface of algae such as *Phyllophora* and *Ulva* and also in sediments due to sedimentation of the detritus. In addition to *Azotobacter*, *Clostridium* was found attached to algae but these organisms were present in greatest numbers in sediments. Pshenin's report did not include *in situ* values for nitrogen fixation but he did quote fixation rates obtained on laboratory isolates. *Azotobacter* spp. from water and sediment samples and from *Phyllophora* and *Clostridium* from water and sediment samples appeared capable of nitrogen fixation. These results must, however, be viewed with some caution since the method used for measuring nitrogen fixation was nitrogen gain from "nitrogen-free media" measured by the Kjeldahl technique and the "nitrogen-fixing organisms" quoted included the yeasts *Torulopsis* and *Rhodotorula*. Pshenin, however, did establish that potential nitrogen-fixing bacteria do exist in marine waters and sediments although whether they fixed a significant quantity of nitrogen under these conditions is in doubt. Brezonik and Harper (1969) studied nitrogen fixation by presumptive heterotrophic organisms in Lake Mary, Wisconsin, and Lake Mize, Florida, using the acetylene reduction technique. At the time of sampling both lakes were anoxic below a depth of 5 m. In Lake Mary the rate of acetylene reduction in the upper water, where blue-green
algae were not apparent, was only slightly higher than the detection limit of the procedure used. Rates were higher at a depth of 10 m, however, and there was a marked increase in rate at the bottom of the lake (20 m). In Lake Mize the fixation rate was higher in samples collected in July than in August and the reasons for this were not apparent. In both sets of samples, however, fastest rates of acetylene reduction occurred below 9 m. The fastest rates reported were 308 ng nitrogen fixed per litre per hour (which is much lower than those for fixation by blue-green algae in other lakes (see below)). In Lake Erie (Howard et al., 1970) nitrogen fixation in the water column was detected only in the presence of a bloom of blue-green algae but in the sediments the rate was not subject to seasonal variations. Sediment nitrogen fixation occurred only at a low rate and was attributed to heterotrophic bacteria since it occurred in the dark. Keirn and Brezonik (1971) reported positive acetylene reduction in sediments from 7 out of 25 lakes studied in Florida and in sediments from 3 lakes in Guatemala. In the same report these authors confirmed the earlier results from Lake Mize, Florida (Brezonik and Harper, 1969), and isolated 3 bacterial species capable of nitrogen fixation. These were reported to be Clostridium sp. and purple sulphur bacteria of the genera Thiospirillum and Chromatium. A nitrogen-fixing Clostridium sp. has also been isolated from the Waccasassa estuary on the Florida coast (Brooks et al., 1971) where bacterial nitrogen fixation is reported to occur within the top 2–5 cm of the sediments. Werner et al. (1974) recently reported the isolation of two different facultatively anaerobic bacteria from sea water and sediments off the Oregon coast. These authors referred to these bacteria as Klebsiella pneumoniae and Enterobacter aerogenes and showed that they were capable of nitrogen fixation under anaerobic conditions in the dark when grown in a “natural marine community” in a laboratory model ecosystem. Whether these bacteria are of significance in a natural environment must await in situ studies.

Fixed nitrogen sources such as nitrate and ammonia are potent repressors of the synthesis of nitrogenase in bacteria while nitrogen itself is not probably required for enzyme synthesis (Wilson, 1958; Hill et al., 1972; Dalton and Mortenson, 1972; Benemann and Valentine, 1972; Drozd et al., 1972; Tubb and Postgate, 1973). Several factors control the rate of bacterial nitrogen fixation in aquatic environments including the provision of carbon and energy sources, the oxygen
tension and the concentration of fixed nitrogen sources. With *Azotobacter* spp. the requirement for carbon source and the oxygen tension are closely linked since for nitrogen fixation to occur to any degree under aerobic conditions there must be an abundance of carbon source to satisfy the requirements of respiratory protection (Dalton and Postgate, 1969a, b). It seems very unlikely that an aquatic environment will furnish sufficient carbon source to enable *Azotobacter* to fix appreciable quantities of nitrogen except at low oxygen tensions. In the case of the anaerobic bacteria such as *Clostridium* spp. the oxygen tension is of obvious significance. Microenvironments may occur, however, with localized conditions of oxygen depletion. For example Line and Loutit (1973) reported positive "aerobic" acetylene reduction with *Clostridium* on agar slopes in the presence of a variety of aerobic organisms. In particular the presence of *Pseudomonas azotogensis* with the *Clostridium* gave marked acetylene reduction rates. Since in any natural environment mixed populations will occur, then this type of association may be common. Nitrogen fixation only occurs in the absence or in the presence of only low concentrations of fixed nitrogen, for example Drozd et al. (1972) showed that the synthesis of nitrogenase in sulphate-limited cultures of *Az. chroococcum* did not occur when ammonia was present in the system in detectable quantities. After the removal of ammonia repression the synthesis of nitrogenase was rapid, being complete within 75 per cent of the population doubling time. While the principal effect of adding ammonia to cultures fixing nitrogen was a cessation of nitrogenase synthesis there was also an effect on enzyme activity (30 per cent decrease with or without a lag; Hardy et al., 1968; Shah et al., 1972; Drozd et al., 1972). In *Klebsiella pneumoniae* again grown in sulphate-limited cultures (Tubb and Postgate, 1973), nitrogenase activity was proportionately repressed with increasing concentrations of ammonia in the incoming medium. On de-repression, following exhaustion of ammonia, the synthesis of nitrogenase lagged for 90 minutes, but was complete within the doubling time of the culture. In *Klebsiella pneumoniae* and in * Clostridium pasteurianum* the effect of ammonia was to repress nitrogenase synthesis and, unlike the situation in *Azotobacter chroococcum*, it had little, if any, effect on nitrogenase activity, which was therefore diluted out during growth of the culture on ammonia (Daesch and Mortenson, 1972; Mahl and Wilson, 1968; Tubb and Postgate, 1973).

Of particular significance in relation to ammonia repression of
nitrogen fixation in aquatic environments was the report by Hill et al. (1972) that in chemostat cultures of *Azotobacter chroococcum* the degree of repression produced by a particular concentration of ammonia was a function of the population density of the culture. Thus in populations of low cell density, only low concentrations of ammonia were required to repress nitrogenase synthesis. It will be of considerable significance to ascertain whether this effect is confined to *A. chroococcum* or more widespread in nitrogen fixing bacteria. Hill et al. (1972) also showed that in *A. chroococcum* nitrogen fixation and ammonia utilization probably occurred at the same time, provided the ammonia concentration was sufficiently low (in their chemostat cultures the residual ammonia levels were below the sensitivity of the assay system used). Once again it is not apparent how widespread is this effect.

3.1.2 *Nitrate reduction*

Most of the literature on this subject is concerned with dissimilatory (respiratory) reduction, in which nitrate serves as an alternative electron acceptor to oxygen, and comparatively little is known of the assimilatory process. Aspects of dissimilatory reduction and the overall process of denitrification has been reviewed in some detail by Painter (1970), by Payne (1973) and by Keeney (1972) who has discussed this process in relation to nitrogen turnover in lake sediments. Dissimilatory reduction may, in part, be responsible for the secondary nitrite maximum found in oxygen depleted waters (see section 1) and this nitrite possibly serves as an assimilatory nitrogen source under some conditions. A list of chemosynthetic microorganisms capable of utilizing nitrate as sole nitrogen source are included in Payne's (1973) review. The development of enzymes necessary for this assimilatory reduction may occur either aerobically or anaerobically. In general, assimilatory nitrate reductases are soluble and unable to reduce chlorate. Enzymes of this type have been detected in a number of bacteria including *Pseudomonas* spp., *Micrococcus denitrificans*, *Bacillus* and *Hafnia*.

The physiology of nitrate assimilation in a marine pseudomonad (strain PL) has been studied in our laboratory. Since small quantities of nitrite are often found in the spent medium of early logarithmic phase batch cultures growing on nitrate and organisms capable of growth on nitrate are also able to grow, without lag, on nitrite it appears likely that the assimilatory reduction in marine pseudomonads proceeds
via nitrite to ammonia. Only very small quantities of ammonia have ever been detected in nitrate grown cultures of these organisms and these only in intracellular pools (extracted with hot water). This is true both of batch and chemostat cultures grown on limiting or excess quantities of nitrate. In the latter case small amounts of nitrite may be found intracellularly but the bulk of the nitrogen excess remains in the culture medium as nitrate and there is no tendency for the cells to accumulate nitrite or ammonia in significant concentrations (Brown et al., 1972, 1973b). This suggests that growth on nitrate is, physiologically, equivalent to nitrogen limitation with some “in-built” limitation occurring either at the level of nitrate uptake or nitrate reductase. This phenomenon is common to Pseudomonas aeruginosa and Ps. fluorescens in addition to a number of marine pseudomonads. In the marine pseudomonad PL₁ a soluble activity catalysing the NADH-dependent reduction of nitrate to nitrite has been detected and appears to be as assimilatory nitrate reductase (D. S. Macdonald-Brown and C. M. Brown, unpublished). The highest enzyme activities were found in nitrate-limited cultures and in nitrogen-limited cultures in which both nitrate and ammonia served as sources of nitrogen. Lower activities were found in cultures grown in the presence of excess nitrate (either carbon or phosphate limited) and in ammonium or glutamate (nitrogen) limited cultures. An excess of ammonium or glutamate appeared to repress enzyme synthesis although traces of enzyme activity were detected in those cultures with such a nitrogen excess and also containing nitrate. This observation indicates that the synthesis of nitrate reductase in this organism does not require the presence of nitrate but will proceed in its absence provided that only low or limiting concentrations of alternative nitrogen sources are present. High concentrations of ammonia inhibited nitrate uptake in strain PL₁ and ammonia was therefore used preferentially. This preferential uptake, however, only occurred when the ammonia concentration was greater than $1 \times 10^{-3}$ M and below that concentration both ammonia and nitrate were utilized at the same time.

In Azotobacter chroococcum the assimilatory nitrate reductase was synthesized in the absence of nitrate and in the presence of dinitrogen and ammonia but highest activities were found in cultures growing on nitrate or nitrite. Cultures containing KNO₃ had almost three times the nitrate reductase activity of those grown on NH₄NO₃ (Guerrero et al., 1973). Nitrite reductase from this organism (Vega et al., 1973)
is an adaptive enzyme whose formation required the presence of either nitrate or nitrite in the medium. Ammonia in the culture medium had little effect on the cellular activity of nitrite reductase and it was suggested that *A. chroococcum* could utilize nitrate or nitrite in the presence of ammonia although little evidence was advanced to support this suggestion.

### 3.1.3 Ammonia assimilation

It is well established that the assimilation of ammonia in bacteria occurs largely either via glutamate dehydrogenase (GDH) or glutamine synthetase/glutamate synthase (GS/GOGAT), depending on the nature and concentration of the medium nitrogen source, and that glutamate is the net product of the amination reactions (see above, Tempest et al., 1973; Brown et al., 1974). It is pertinent to establish the relative significance of these alternative mechanisms in aquatic bacteria that are capable of growth on atmospheric nitrogen, nitrate or ammonia.

In relation to ammonia assimilation during nitrogen fixation there is some doubt as to the route followed. From theoretical considerations it would be appropriate that assimilation should proceed through GS/GOGAT in order to maintain the intracellular concentration of ammonia at a low level since this compound is a potent regulator of nitrogen fixation (Benemann and Valentine, 1972; Dalton and Mortenson, 1972). Experimental evidence for such a system, however, is not clear cut, except in the case of *Clostridium pasteurianum* which according to Dainty and Peel (1970) lacks GDH and therefore relies solely on GS/GOGAT for ammonia assimilation (Dainty, 1972). Whether or not this simple state of affairs operates in all clostridia is not known. According to Nagatani et al. (1971) the presence of GOGAT is obligatory for ammonia assimilation in a number of nitrogen-fixing bacteria including *Klebsiella pneumoniae*, *Azotobacter vinelandii*, *Clostridium pasteurianum*, *Chromatium vinosum* strain D, *Chlorobium thiosulphatophilum* and *Rhodospirillum rubrum*. These authors also showed that in *K. pneumoniae* the ratio GOGAT/GDH was much higher in cultures grown on atmospheric nitrogen than in those grown on ammonia. No such variations were apparent with *Cl. pasteurianum* and *Chromatium*. As pointed out by Drozd et al. (1972) these GOGAT/GDH ratios may prove to be of doubtful value since the enzyme assays were obtained from batch cultures grown under rather ill-defined conditions. While
this is undoubtedly true, these results do indicate the presence of GOGAT in these nitrogen-fixing bacteria. In *K. pneumoniae* the GS activity of cells grown on atmospheric nitrogen was high while no activity was detected in cultures grown on high concentrations of ammonia. Therefore, in this organism, at least, cultures grown on atmospheric nitrogen contained both GS and GOGAT and, moreover, the GS from these cells had a low level of adenylylation and therefore a very high affinity for ammonia. Perhaps the best evidence for the involvement of GS/GOGAT in *K. pneumoniae*, however, was the fact that mutants that failed to grow on fixed atmospheric nitrogen or in media containing only low levels of fixed nitrogen contained only low GOGAT activities while GS and GDH activities remained near normal. These mutants grew normally on high concentrations of ammonia when this substrate was assimilated, presumably, via GDH. A recent report of Slater and Morris (1974) extends the observations of Nagatani *et al.* (1971) with *R. rubrum* in which the synthesis of glutamate was apparently light dependent under some cultural conditions. These authors suggest that these results indicate that light-generated ATP was required for the operation of the GS/GOGAT pathway. The evidence presented is, however, circumstantial and hinges to a considerable extent on the proposed correlation between the presence in the amino acid pool of significant quantities of glutamine and glutamate and the operation of the GS/GOGAT pathway in marine pseudomonads (Brown and Stanley, 1972; Brown *et al.*, 1972). It must be emphasized, however, that while this correlation remains good for marine pseudomonads it has yet to be shown to be true for any other microorganism. In well-defined experiments with *Az. chroococcum* grown in sulphate-limited chemostats with alternatively atmospheric nitrogen, nitrate and ammonia as nitrogen source, Drozd *et al.* (1972) reported no variation in GDH or GOGAT activities and, therefore, no variation in GOGAT/GDH ratio with nitrogen source. Unfortunately no data regarding GS were reported and since control of ammonia assimilation is often exerted through either the activity or synthesis of this enzyme (or both) the assimilatory route followed by ammonia in *Az. chroococcum* during nitrogen fixation remains in doubt.

During the growth of *Pseudomonas* spp. on nitrate, ammonia assimilation appears to proceed via GS/GOGAT. A preliminary survey of a number of marine pseudomonads (Brown *et al.*, 1972) isolated from coastal waters showed that all organisms studied contained GOGAT
activity and, in addition, many also contained NAD-linked GDH when grown on nitrate. The GOGAT activities were higher in cells grown on nitrate than in those grown on casamino acids while the reverse was true of the GDH. Experiments with chemostat cultures of a number of these organisms established that the activities of both GS and GOGAT were high in those cells grown on nitrate irrespective of the nitrate concentration. Since, as discussed above, these nitrate-grown cultures behaved as though nitrogen limited, the ammonia level in the culture was never sufficient to allow any significant assimilation via GDH. Therefore these pseudomonads, when grown on nitrate, assimilated ammonia via GS/GOGAT. Similar results were obtained with *Ps. aeruginosa* and *Ps. fluorescens* (Brown et al., 1972, 1973b). It is not apparent, at the present time, to what extent this “self limitation” is found in bacteria. It appears to operate in *K. aerogenes* in a manner similar to the pseudomonads but not, for example, in cultures of *Rhizobium leguminosarum* (Brown and Dilworth, 1974) in which an excess of nitrate appeared to be assimilated via GDH. Since nitrate is known to be as potent a repressor of nitrogenase as ammonium itself in *Az. chroococcum* then this organism probably behaves as does *R. leguminosarum*.

The concentrations of ammonia in most unpolluted aquatic environments are likely to be low and ammonia assimilation under these conditions is most likely to proceed via GS and GOGAT in those organisms containing these enzymes (Tempest et al., 1973; Brown et al., 1974). GS from *E. coli* is probably the best studied enzyme involved in bacterial nitrogen metabolism and the elegant studies of Stadman and Holzer and their respective colleagues has been the subject of a number of reviews (for example, see Holzer et al., 1969; Shapiro and Stadman, 1970; Prusiner et al., 1972). In essence, in *E. coli* the highest GS activity and that with the highest substrate affinity is to be found in cultures growing in the presence of low ammonia concentrations where it is admirably suited to “scavenge” this substrate (Umbarger, 1969). It is not apparent, however, whether modulation of GS activity occurs to any extent in those bacteria common in natural aquatic environments although evidence to this effect is available for *Klebsiella* spp. (Nagatani et al., 1971) and *Bacillus* spp. (Deuel and Prusiner, 1974; Hubbard and Stadman, 1967a, b). Evidence for the operation of GS/GOGAT, however, is more readily available. GOGAT was first discovered in extracts from ammonia-limited cultures of *Klebsiella aerogenes* (Tempest et al., 1970) which also contained high levels of GS activity (Meers and
Tempest, 1970) but little GDH activity (Meers et al., 1970) and more-
over the $K_M$ of the GDH for ammonia was such as to preclude its
operation in an ammonia-limited environment. In glucose-limited
cultures containing an excess of ammonia neither GS nor GOGAT
activity was recorded while the GDH level was very high, indicating
that under these conditions ammonia was assimilated via GDH. The
role of GS/GOGAT in ammonia assimilation in K. aerogenes has been
emphasized by the report that mutant organisms lacking GOGAT
(but containing GDH) failed to grow on ammonia at concentrations
less than $1 \times 10^{-3}$ M but grew normally on higher concentrations of
ammonia (Brenchley et al., 1973). In Escherichia coli the synthesis of
both GOGAT and GDH were unaffected by the medium ammonia
concentration and it is assumed that in this organism GS/GOGAT is
operative at low ammonia concentrations due to the high affinity of
GS for ammonia (Berberich, 1972). It appears, also, that GS/GOGAT
operates in ammonia assimilation in Bacillus spp. in much the same
fashion as in K. aerogenes and E. coli (Elmerich and Aubert, 1971;

In the marine pseudomonads, GS, GOGAT and NAD-linked GDH
activities were detected in cells grown under ammonia limitation
(Brown et al., 1972, 1973b) but the virtual absence of ammonia in
either the medium or the intracellular pools of these organisms
indicated that GDH could not contribute significantly to ammonia
assimilation due to its low substrate affinity. Thus assimilation was
considered to proceed via GS and GOGAT. That the GS of these
organisms was able to “scavenge” ammonia was evident from its
high substrate affinity ($K_M$ of 0-3 mM) but it was also apparent that the
efficient use of the GS/GOGAT pathway required a high intracellular
glutamate concentration since the $K_M$ for glutamate of the GS from
strain PL$_1$ was about 20 mM. Growth on an excess of ammonia resulted
in a much reduced GS activity in strain PL$_1$ and a lack of detectable
activity in strain SW$_2$. Whether these changes reflect changes at the
level of enzyme synthesis or modulation of enzyme activity is unknown.
Cultures grown on an ammonia excess also contained NADP-linked
GDH activity in addition to that linked to NADH. These two GDHs
showed different pH and temperature characteristics and appeared to
be distinct iso-enzymes. Collectively they may account, at least in part,
for ammonia assimilation in the presence of an ammonia excess and in
strain PL$_1$ there must be competition for substrate between the high
affinity, ATP requiring GS/GOGAT system and the low affinity GDHs under these conditions. Other marine pseudomonads and *Ps. fluorescens* behave in a similar fashion to strain PL₃.

### 3.2 BLUE-GREEN ALGAE

Blue-green algae are located ubiquitously in aquatic habitats, being found under a variety of conditions of temperature, salinity and nutrient concentrations. According to Fogg *et al.* (1973) only three limitations are apparent: (a) most species are obligately phototrophic and require light; (b) neutral or alkaline conditions are preferred; and (c) with the exception of the genus *Trichodesmium* (which may form vast oceanic blooms) and to a lesser extent *Calothrix* sp., blue-green algae are generally absent from the open sea. There are probably, in the physiological sense, two reasons for the otherwise broad ecological distribution of blue-green algae. First, being photosynthetic organisms they can be, and very often are, independent of the exogenous carbon sources required by heterotrophic organisms. Second, in general, blue-green algae are extremely versatile with respect to nitrogen requirements, being able to utilize preformed sources of fixed nitrogen both organic and inorganic (nitrate and ammonia), whilst many species are independent of fixed nitrogen sources in that they are able to fix atmospheric dinitrogen by the mechanisms described previously. The various physiological parameters which may influence nitrogen assimilation in blue-green algae will be considered here.

#### 3.2.1 Nitrogen fixation

An important factor which could influence nitrogenase activity is the availability of its substrate dinitrogen. The $K_M$ values of nitrogenase in cell-free extracts for dinitrogen fall within the range 0.002–0.006 atmospheres (Fogg *et al.*, 1973), whilst Ohmori and Hattori (1973) have reported that the nitrogen-fixing system in whole cells of *Anabaena cylindrica* is half saturated at a partial pressure of 0.2 atmospheres. This means, presumably, that a dinitrogen gradient exists between the outside of the cell and the site of nitrogenase. In view of the low $K_M$ values for dinitrogen, and the solubility of the gas in natural waters (see above) it is unlikely that blue-green algal nitrogen fixation is limited by the availability of the substrate. The role of dinitrogen as a possible
inducer of nitrogenase is not clear. Ohmori and Hattori (1973) state that if dinitrogen does induce the enzyme, then it is fully induced at a partial pressure of 0.0001 atmospheres. If this were the case, then in virtually all aquatic environments the levels of dinitrogen present would be such as to place no restriction upon the synthesis of the enzyme. However, it is equally possible that the synthesis of the enzyme is controlled by the repressive effect of certain compounds, and in their absence synthesis is derepressed.

Oxygen is known to be a potent inhibitor of nitrogenase (see above), while Bone (1971b) has shown that it also represses the synthesis of the enzyme. In filamentous forms nitrogenase is believed to be compartmentally protected in heterocysts and thus one might expect oxygen tension \( (pO_2) \) to exert little effect upon the natural distribution of these forms. Nonheterocystous algae, e.g. Gloeocapsa, Plectonema, fix nitrogen best under microaerophilic conditions, and the optimum \( pO_2 \) for such organisms might be expected to be that which allows respiration to proceed but which is insufficient to inhibit nitrogen fixation. On the basis of reduced oxygen tension with depth, therefore, one might predict that increasing depth would have little effect upon nitrogen fixation by heterocystous strains, whilst fixation by nonheterocystous strains would be more efficient further from the highly oxygenated surface layers. In fact, it would appear from the work of Stewart and Pearson (1970) that blue-green algae in general grow better under microaerophilic conditions than under fully aerobic ones. Blooms of algae become prominent in lakes which show summer oxygen depletion of the hypolimnion (Zimmerman, 1969), but any tendency for population maxima to occur at a particular value of \( pO_2 \) may well be confused by the production of oxygen by the algae themselves, and so such information must be interpreted with caution. Further, the optimal depth at which an alga grows really represents the resultant of response to a variety of other factors, e.g. light intensity, concentration of other nutrients, inherent buoyancy.

Two important requirements for the fixation of nitrogen are an energy source and a source of reducing power. There is good evidence that photophosphorylation supplies energy to phototrophically growing algae (Cox and Fay, 1969; Fay, 1970). The situation with respect to photoreducing power is less clear; the role of photoreduced ferredoxin as source of reducing power has been demonstrated \emph{in vitro} (Bothe, 1970; Smith and Evans, 1971; Smith \emph{et al.}, 1971). However, this does
not necessarily confirm a physiological role (see Fogg et al., 1973), and furthermore photosystem II activity is lacking from heterocysts, which are implicated as the sites of nitrogen fixation at least in those algae which possess them. Blue-green algae may grow, and fix nitrogen, heterotrophically in the dark, although these processes are slow in comparison with light grown organisms. It is considered that under dark conditions oxidative phosphorylation supplies energy and low dark respiration rates are responsible for the low dark rates of nitrogen fixation. The source of dark-generated reducing power is uncertain but pyruvate has been implicated in the process (Cox, 1966; Fay and Cox, 1966; Cox and Fay, 1967; Cox and Fay, 1969; Leach and Carr, 1971). Thus the availability of light may play an important physiological role in nitrogen fixation both directly as a source of energy and reducing power, and indirectly via the production of oxygen. Such a physiological role could therefore play a part in the distribution of blue-green algae in aquatic ecosystems, and, indeed, photic distribution of blue-green algae is observed. Further, since blue-green algal blooms represent a major site of nitrogen input into aquatic ecosystems, their distribution might well determine the distribution of other organisms. However, care should be exercised in the relation of distribution patterns of organisms to a single parameter, since such patterns must represent the resultant of the responses of the organisms to all of the environmental parameters in a particular location.

The influence of salinity upon nitrogen fixation does not appear to have been investigated to any extent. This is rather surprising since this could well influence the nitrogen balance of ecosystems, particularly in those environments which are subject to large variations in salinity, e.g. estuaries and salt marshes. One might observe substantial changes in blue-green population patterns depending upon whole cell response to salt, with resultant changes in the total nitrogen fixed, depending upon the relative nitrogen-fixing ability of different organisms, or salinity variations may govern either the synthesis or activity of nitrogenase itself. It would seem, therefore, that the time is ripe for knowledge in this field of almost total ignorance.

Another factor that may exert an effect upon nitrogen fixation by blue-green algae is the presence and concentrations of fixed nitrogen sources, the principal fixed nitrogenous compounds in aquatic ecosystems being nitrate and ammonia. Such fixed nitrogen could exert an effect at two levels, namely on enzyme activity and on enzyme
synthesis. The available evidence suggests that fixed nitrogen sources show little effect upon the activity of preformed nitrogenase. Stewart et al. (1968) showed that in *Nostoc muscorum* 7·2 mm of nitrate–nitrogen did not inhibit the activity of nitrogenase, whilst Dharmawardene and Stewart (unpublished, cited by Fogg et al., 1973) have failed to demonstrate inhibition of nitrogenase activity using up to 14·4 mm of ammonium nitrogen. However, there is evidence to suggest that fixed nitrogen sources repress the synthesis of nitrogenase. Bone (1971a) has demonstrated that the synthesis of nitrogenase in *Anabaena flos-aquae* is repressed 24-fold by $15 \times 10^{-8} \text{M}$ potassium nitrate, whilst Ohmori and Hattori (1973) failed to detect repression of nitrogenase in *Anabaena cylindrica* using nitrate concentrations up to $2 \times 10^{-2} \text{M}$. The latter workers also showed that ammonia concentrations of $10 \times 10^{-3} \text{M}$ completely repressed the formation of nitrogenase but had no effect upon the activity of preformed enzyme (see also Stewart, 1973). There is a growing body of evidence that the intracellular free amino acid pool may regulate the rate of synthesis of nitrogenase (Stewart et al., 1968; Jewell and Kulasoorija, 1970; Neilson et al., 1971; Streicher et al., 1971). Further, there is a good deal of evidence which indicates that the development of heterocysts is prevented by the presence of combined nitrogen (Fogg, 1942, 1944; Fay et al., 1964; Mickelson et al., 1967; Stewart et al., 1968; Ogawa and Carr, 1969). Since heterocysts are implicated as the cellular location of nitrogenase and the development of heterocysts is paralleled by the appearance of the enzyme, such inhibition of heterocyst differentiation presumably also represents reduced levels of synthesis of nitrogenase.

Given, therefore, that fixed nitrogen sources can influence the synthesis of nitrogenase (and may modulate its activity), the pertinent question is whether, in aquatic ecosystems, sufficiently high concentrations of fixed nitrogen sources are present to exert such effects. In the majority of eutrophic freshwater habitats, the level of fixed nitrogen seldom exceeds $1·07 \times 10^{-4} \text{M}$ nitrogen and is substantially lower than this in an oligotrophic environment (see Introduction; Vollenweider, 1968; Keeney, 1972), although restricted, localized concentrations may build up. Similarly, low levels of combined nitrogen have been reported for hot springs which support the growth of blue-green algae (Castenholz, 1969; Stewart, 1970b). The question of the concentration of fixed nitrogen sources in marine environments with respect to nitrogen fixation have been considered by Stewart (1971), and it would appear
that even taking into account areas of localized concentration, the levels of fixed nitrogen are insufficient, by several orders of magnitude, to influence the synthesis of nitrogenase. Thus in the vast majority of aquatic habitats, fixed nitrogen probably exerts little influence upon the synthesis or activity of nitrogenase. However, such fixed nitrogen sources may exert an effect upon the total nitrogen-fixing capacity of a particular location inasmuch as such sources will allow the growth of non-nitrogen-fixing organisms, resulting in competition for nutrients with a possible reduction, therefore, in blue-green algal numbers.

Since molybdenum and iron are essential components of the nitrogenase complex, it is pertinent to examine the concentrations of these two elements in aquatic ecosystems to determine whether they might be limiting with respect to enzyme synthesis. The optimum concentration of molybdenum reported for nitrogen fixation in various blue-green algae is about 0.1 mg l\(^{-1}\) (Wolfe, 1954; Arnon, 1958; Okuda \textit{et al.}, 1962; Goriunova and Maksudov, 1972) and as the concentration of molybdenum in most aquatic environments approaches this figure, under most conditions molybdenum would not be limiting with respect to nitrogen fixation. The case of iron is complicated by the fact that iron is required for a variety of cellular molecules and processes. The average total iron content of sea water is about 0.01 mg l\(^{-1}\) (Goldberg, 1965) whilst the optimal iron concentration required for nitrogen fixation is about 0.1 mg l\(^{-1}\) (Stewart, 1971) so that in marine ecosystems, at least, iron could possibly limit nitrogen fixation.

Recently, Horne and Goldman (1974) have investigated the effects of copper upon the fixation of blue-green algae from the eutrophic Clear Lake, California. They found that the addition of 5 \(\mu\)g l\(^{-1}\) copper to lake water samples, the predominant nitrogen-fixing genus of which was \textit{Anabaena}, reduced nitrogen fixation by 90 per cent within three days whilst \(^{14}\)C\(\text{O}_{2}\) uptake was only inhibited by approximately 10 per cent in this time. They also showed that different blue-green algae respond to different degrees, \textit{Anabaena} growth being less affected than \textit{Aphanizomenon} growth at 5 \(\mu\)g l\(^{-1}\) Cu. However, these findings do not necessarily indicate that nitrogen fixation is inhibited to the same extent \textit{in situ}, since the majority of copper may be chelated under such conditions. While the molecular basis of such inhibition is unknown, the important fact remains that copper may well modulate nitrogen fixation in aquatic ecosystems. The average copper content of non-polluted fresh and saline waters is about 5 \(\mu\)g l\(^{-1}\) (see Riley and Chester,
1971), and since the equilibrium free Cu⇌chelated Cu probably lies well to the right, the effects of such copper are probably controlled by the availability of chelating agents. However, in waters polluted by copper compounds, e.g. the fungicide and algicide copper sulphate, the total nitrogen fixed and thus the nitrogen balance of the ecosystem may well be controlled by the concentration of copper.

Goriunova and Maksudov (1972) have demonstrated a cobalt requirement for blue-green algal nitrogen fixation, and in some aquatic locations, it is possible that cobalt could be limiting.

So far as the total nitrogen fixed by blue-green algae in a particular environment is concerned, it is necessary to question which nutrient(s) might be limiting, since if such limitation were relieved, then growth would proceed with a resultant increase in nitrogen fixation until the same, or another, nutrient became limiting. Since the blue-green algae can be true autotrophs, in the sense that they may fix both carbon and nitrogen, it has been assumed that in many natural environments phosphorus is limiting, and when partial eutrophication of an oligotrophic environment takes place, the phosphorus limitation is relieved and an algal bloom occurs until phosphorus again becomes limiting. Thus phosphorus may well control the total fixation of nitrogen in a particular environment. There is also evidence to suggest that a depletion of phosphorus may influence the rate of nitrogen fixation directly. Stewart et al. (1970) have shown that when phosphorus-starved *Anabaena flos-aquae* is supplied with as little as 3–10 μg l⁻¹ phosphorus, there is a rapid marked stimulation in nitrogenase activity. Similar results were obtained by Stewart and Alexander (1971), and these authors further showed that endogenous ATP levels increased substantially when phosphorus limitation was relieved. On the basis of these results it is suggested that nitrogen fixation could be limited in natural environments via a depletion in cellular ATP (see Fogg et al., 1973).

Many blue-green algae are able to grow in the dark, or light restricted conditions, by heterotrophic utilization of organic carbon compounds. Thus under conditions where phototrophic growth would not be possible, or under conditions where a degree of heterotrophic growth may complement phototrophic growth, the presence of organic compounds may exert profound effects upon the nitrogen fixed by a given population. In situations where eutrophication of an oligotrophic water takes place in which organic compounds and fixed inorganic
nitrogenous compounds are added simultaneously, one might expect to see opposing effects upon nitrogen fixation, and indeed this was observed by Horne and Fogg (1970) in studies involving lakes in the English Lake District. This kind of mechanism could well be operative in most aquatic environments, with the probable exception of the open ocean.

Bearing in mind the possible physiological influence of all of the previously mentioned factors, to what extent may blue-green algal nitrogen fixation contribute to the nitrogen content of aquatic ecosystems? Two techniques have been principally employed: (a) the use of $^{15}$N as tracer, and (b) the acetylene reduction technique. In most natural waters, blue-green algae are by far the most quantitatively important nitrogen-fixing organisms, and in many cases are assumed to be the sole organisms responsible for observed fixation. Such *in situ* estimates of nitrogen fixation are subject to very large error values since the sampling techniques, however careful, can rarely be absolutely representative of a particular location. However, since blue-green algae are predominantly phototrophic organisms one might expect nitrogen fixation to be predominantly confined to surface layers, and indeed this has been demonstrated many times. Much of the data available up to the present time relates to rates of nitrogen fixation (or acetylene reduction) in samples from such surface waters.

Dugdale *et al.* (1959), using $^{15}$N, were the first to present conclusive evidence demonstrating the fixation of nitrogen in natural waters, and subsequently many freshwater locations, principally lakes, have been investigated. Dugdale and Dugdale (1962) reported seasonal variations in nitrogen fixed with maximum rates of about 130 $\mu$g N l$^{-1}$ day$^{-1}$ during the summer period, probably due to higher temperatures and longer periods of daylight, and similar results were obtained on studies on Wisconsin lakes by Goering and Neess (1964). Granhall and Lundgren (1971) estimated that nitrogen fixation by blue-green algae (principally *Aphanizomenon* sp.) in Lake Erken, Sweden, was responsible for an annual contribution of 0.5 g N m$^{-2}$. Horne and Viner (1971) have studied nitrogen fixation in Lake George, Uganda, recorded rates of about 40 $\mu$g l$^{-1}$ day$^{-1}$, and suggested that nitrogen fixation is responsible for approximately 60 per cent of the annual total nitrogen gain by this lake. This figure contrasts with a calculated contribution of 14 per cent for Lake Mendota, Wisconsin (Lee, 1966; cited by Keeney, 1972), whilst Horne and Goldman (1972) obtained a figure of
43 per cent for Clear Lake, California. Data such as these demonstrate that the relative importance of nitrogen fixation with respect to nitrogen balance may vary widely with the variable nature and degree of environmental parameters of different locations. It should be mentioned also that figures such as those above take no account of the possible contribution of bacterial nitrogen fixation in sediments, a subject about which little is known (see above).

There is a considerable, and ever growing, body of evidence to suggest a relationship between surface nitrogen fixation (presumably largely algal) and eutrophication in lakes. In a survey of Wisconsin lakes Rusness and Burris (1970) were able to correlate increased rates of nitrogen fixation with the degree of eutrophication of the site, and similar results, although with lower levels of nitrogenase activity, were obtained in another survey of Wisconsin lakes by Stewart et al. (1971). Horne and Fogg (1970) in a study of the English lakes obtained results which led them to suggest that optimal nitrogen fixation takes place at an optimal intermediate degree of eutrophication, and this serves only to stress the complexity of interacting factors that may influence the growth of, and fixation of nitrogen by, blue-green algae. A study in Clear Lake, California, by Horne et al. (1972) is particularly interesting in this respect. These workers studied the autumnal bloom of *Anabaena circinalis* and found that the fixation of nitrogen could be correlated with at least five factors, namely:

i. large numbers of heterocysts;
ii. low concentrations of nitrate;
iii. low concentrations of ammonia;
iv. high concentrations of phosphate; and
v. moderately high concentrations of organic nitrogen.

There is a dearth of information pertaining either to the rate of, or ecological significance of, blue-green algal nitrogen fixation in flowing freshwaters, although a variety of organisms may be isolated from such sites.

There are several reports of algal nitrogen fixation in relatively warm aquatic habitats (Billaud, 1967; Stewart, 1968, 1970a, b; Castenholz, 1969), with the optimum temperature range 25–54 °C, although Stewart and his collaborators have detected nitrogenase activity at 60 °C in a population dominated by *Mastigocladus* (cited by Fogg et al., 1973).
Many blue-green algae isolated from saline environments have been reported to fix nitrogen (for summary see Stewart, 1971), but the predominant genera in such habitats appear to be *Trichodesmium* and *Calothrix*. The first report of *in situ* nitrogen fixation by *Trichodesmium* populations was that of Dugdale *et al.* (1961), whilst Dugdale *et al.* (1964) showed fixation rates from various locations of about 2 μg l⁻¹ h⁻¹. *Trichodesmium* species have also been implicated in *in situ* nitrogen fixation in many locations (Goering *et al.*, 1966; Bunt *et al.*, 1970; Carpenter, 1973; Taylor *et al.*, 1973).

There have been few reports of nitrogen fixation in estuarine environments. In one such study on the Florida Gulf Coast, Brooks *et al.* (1971) detected fixation rates in surface sediments of 0.64–6.0 ng N g⁻¹ h⁻¹, but concluded that this was mainly due to bacteria, probably *Clostridium* species. Stewart (1965, 1967a, b) has studied *in situ* nitrogen fixation by epilithic algae and sand-dune slack algae in east coast locations of England and Scotland. In a rocky shore, fixation (principally by *Calothrix* species) accounted for approximately 2.5 g m⁻² year⁻¹, which is equivalent to 41 per cent of the mean total nitrogen present; seasonal variations in the amounts of nitrogen fixed were observed, the maximum occurring in the period from March to September. The sand-dune slack habitat was subject to blooms of *Nostoc* sp. especially in the spring and autumn, and this was reflected in the nitrogen fixed. In this environment, another parameter is involved in fixation, in that considerable moisture changes can take place and thus the environment can range from truly aquatic (in times of flooding) to terrestrial (in times of desiccation). Stewart points out that this may profoundly affect the nitrogen fixed, and estimated that nitrogen fixation in such a habitat could account for a minimum of 20 per cent of the mean total nitrogen present. In a study of blue-green algae in a salt marsh, Webber (1967) found that about half the organisms present were heterocystous and thus potentially good nitrogen fixers but no quantitative data were given. It was pointed out previously, in connection with the mixing of fresh and saline waters in estuarine environments, that a study into the effects of salt concentration upon nitrogen fixation by blue-green algae would be interesting, and could well be important from the point of view of nitrogen balance in ecosystems where variations in salt concentration occur. In the case of a salt marsh such a consideration could be especially important, in view of the possible extremes of salinity encountered, added to which is the additional
problem of the concentration of organic and/or nitrogenous nutrients available.

3.2.2 Nitrate reduction

By comparison with nitrogen fixation, little is known concerning the physiology of the assimilatory reduction of nitrate in blue-green algae. The $K_M$ of nitrate reductase for nitrate in *Anabaena cylindrica* is $7 \times 10^{-5}$ M (Hattori, 1962a; Hattori and Myers, 1967), a level which was shown by Eppley and Coatsworth (1968) to be equal to the half saturation constant ($K_S$) for nitrate uptake in this organism, whilst the $K_M$ of nitrite reductase for nitrite in this organism is $5 \times 10^{-5}$ M (Hattori, 1962a; Hattori and Uesugi, 1968a). These $K_M$ values are low, and thus one would expect the reduction of nitrate through to ammonia should proceed at a reasonably efficient rate.

Hattori (1962b) first demonstrated the nitrate reducing system in *Anabaena cylindrica*, whilst Ohmori and Hattori (1970) have extended this study by showing that nitrate reductase is fully induced by a nitrate concentration of $2 \times 10^{-2}$ M and that nitrite reductase is fully induced by a nitrite concentration of $0.1 \times 10^{-3}$ M, and further found that nitrate does not repress the synthesis of nitrite reductase. Ohmori and Hattori (1970) have proposed a sequential induction of nitrite reductase by the nitrite produced as product of the nitrate reductase catalysed reaction. Since the concentrations of nitrate and nitrite in nonpolluted environments, even in areas of localized concentration (see, for example, Hattori and Wada, 1971; Wada and Hattori, 1972), seldom, if ever, approach the concentrations mentioned above, it would appear unlikely that either nitrate reductase or nitrite reductase in blue-green algae is ever fully induced, assuming (a) *Anabaena cylindrica* to be typical, and (b) that blue-green algae are unable to concentrate these ions intracellularly to any great extent.

Light-induced reduction of nitrate, nitrite and hydroxylamine was first demonstrated in *Anabaena cylindrica* by Hattori (1962a), and light has been implicated as a source of reducing power for both nitrate and nitrite reductases (Hattori and Myers, 1966; Hattori and Uesugi, 1968b). Thus, in the absence of alternative sources of reducing power, the availability of light could play an important physiological role in blue-green algal assimilatory nitrate reduction in aquatic habitats.

There have, unfortunately, been few reports of the effects of fixed
nitrogen sources, e.g. ammonia upon either the synthesis of or the activity of preformed nitrate and nitrite reductase in blue-green algae. However, one would expect that even if inhibition did take place that in the majority of aquatic environments the levels of such potential inhibitors would be so small as to be relatively noneffective.

It has recently been shown in *Anabaena flos-aquae* (Bone, 1971a) and *Anabaena cylindrica* (Ohmori and Hattori, 1973) that nitrogen may be taken up simultaneously from dinitrogen and nitrate, provided both are supplied in the medium. It would appear, therefore, that nitrogen fixation and nitrate/nitrite reduction *per se* are not mutually repressive, and so in natural aquatic environments the two processes may well take place side by side.

### 3.2.3 Ammonia assimilation

If little was known of the physiology of nitrate reduction in blue-green algae, even less is known about the physiology of ammonia assimilation. This stems from a basic ignorance of the mechanisms by which ammonia is assimilated in these organisms (see above). The possible physiological effects of environmental parameters upon such mechanisms, whatever they may be, are particularly important, not because ammonia may act as a primary source of nitrogen, but because ammonia is an intermediate in the reduction of dinitrogen, nitrate and nitrite. Therefore, if a particular environmental parameter were to affect ammonia assimilation, it would also exert an effect on the reductive processes mentioned above. It is somewhat paradoxical that the mechanisms and physiology of such processes resulting in the formation of ammonia should be reasonably well understood, but that virtually nothing is known of the final step(s) incorporating ammonia into metabolism.

### 3.3 Eukaryotic Microalgae

Since, as discussed above, nitrogen may be a rate-limiting nutrient in aquatic systems, a considerable volume of literature exists in which the kinetics of uptake and assimilation of inorganic nitrogen have been studied in natural populations of phytoplankton and in laboratory cultures in relation to primary productivity. In general, both the uptake of nitrogen and the rate of growth in a nitrogen-limited environment, when measured as functions of nitrogen concentration, produce hyper-
bolic relationships which may be described by a Michaelis–Menten equation: thus \( \mu = \frac{\mu_m [S/K_s + S]}{S} \), where \( \mu \) is growth rate, \( \mu_m \) maximum growth rate, \( S \) the substrate concentration and \( K_s \) a saturation constant. The \( K_s \) or “half saturation constant” gives a convenient measure of the affinity of organism for substrate and is, therefore, of considerable significance in constructing models describing nutrient-limited growth of natural phytoplankton populations and the kinetics of chemostat grown mixed and axenic cultures (Dugdale, 1967; Caperon, 1967, 1968; Eppley and Coatsworth, 1968; MacIsaac and Dugdale 1969; Eppley and Thomas, 1969; Eppley et al., 1970; Thomas, 1970; Caperon and Meyer, 1972a, b; Grenney et al., 1973). It is pertinent at this stage to quote some of the \( K_s \) values obtained with both natural populations and axenic cultures, especially since the work of Eppley and Thomas (1969) indicated that the \( K_s \) values for nutrient uptake and rate of growth for 2 diatoms were very similar. For example, MacIsaac and Dugdale (1969) working with natural marine phytoplankton populations quoted \( K_s \) values for both uptake and growth in the order 0·1 to 10 \times 10^{-6} \text{M} of nitrate and Thomas (1970) values of 0·75 \times 10^{-6} \text{M} for nitrate and 1·5 \times 10^{-6} \text{M} for ammonia. In a natural freshwater population, however, values of 32 \times 10^{-6} \text{M} nitrate were reported (Skelef et al., 1971). This apparent difference between marine and freshwater populations is also evident from reports of \( K_s \) values for isolated organisms and probably reflects the levels of available nutrients in these environments. Thus Eppley and Coatsworth (1968) quoted values for nitrate uptake in the oceanic Chaetoceros gracilis of < 1 \times 10^{-6} \text{M} while the neritic Ditylum brightwellii had a value of 2 \times 10^{-6} \text{M}. Organisms common in estuaries and rock pools (Dunaliella tertiolecta and Phaeodactylum tricornutum) had higher values of about 10 \times 10^{-6} \text{M}, and the highest values quoted were for the freshwater blue-green alga Anabaena cylindrica (70 \times 10^{-6} \text{M} for nitrate, Hattori, 1962a) and Chlorella pyrenoidosa (50 \times 10^{-6} \text{M} for nitrate, Skelef et al., 1971). In a further study of this phenomenon (Eppley et al., 1969b) with oceanic or neritic diatoms and neritic or littoral flagellates it was found that \( K_s \) values for nitrate uptake varied approximately in proportion to cell size and inversely with specific growth rate. Small-celled oceanic species such as Coccolithus huxleyi had the lowest \( K_s \) values (< 0·5 \times 10^{-6} \text{M}) and the flagellate

\(^1\) Droop (1973), however, points out that in a steady-state system uptake measured as increase in, for example, N relative to cell N (rather than to biomass), is itself a growth rate measurement.
Gonyaulax polyedra the highest ($K_s < 5 \times 10^{-6} \text{ M}$). Carpenter and Guillard (1971) showed that differences of the kind quoted above were not confined to variations between species but that clones of the same species had different $K_s$ values depending upon their environment; thus clones isolated from nutrient-poor oceanic waters had $K_s$ values of $< 0.75 \times 10^{-6} \text{ M}$, while the same species taken from an estuarine region had a $K_s$ of $< 1.5 \times 10^{-6} \text{ M}$. It has been suggested that $K_s$ values when taken together with the maximum growth rate of a species may be important factors influencing competition and succession in phytoplankton populations. This has been discussed in some detail by Eppley et al. (1969b) and by Parsons and Takashi (1973) who have shown that in order to predict the outcome of such competition light intensity must also be taken into consideration. This is of especial significance in relation to nitrate and ammonia uptake which are often light dependent. MacIsaac and Dugdale (1972) reported that this light dependence could be described by a Michaelis–Menten expression with a $K_s$ for light intensity ranging from 1 to 14 per cent surface value, a range occurring near to the bottom of the eutrophic zone. The uptake and utilization of nitrogen and therefore the rate of growth in a nitrogen-limited system is therefore a function both of light intensity and nutrient concentration. MacIsaac and Dugdale (1972) concluded that while natural marine phytoplankton populations in oligotrophic waters were certainly nutrient limited, in eutrophic waters light intensity may be a controlling factor with maximum uptake rates occurring near the surface.

A further complication inherent in attempts to predict the behaviour of phytoplankton populations from growth kinetics arises from the observation that in some instances $K_s$ and $\mu_{\text{max}}$ are not constants. During the steady-state growth of a population the rate of nutrient uptake and the rate of cell division are related by the expression

$$Q = \frac{\text{Uptake rate}}{\text{Cell division rate}},$$

where $Q$ is a measure of the nutrient content per cell (Droop, 1968). $Q$ varies with growth rate and has a minimum value when the latter is zero. Caperon (1968) found that the nitrogen content of Isochrysis galbana growing in nitrate varied from 2 to $40 \times 10^{-15}$ mol per cell. Thus, cells accumulated nutrient in excess of the minimum and there was a hyperbolic relationship between this "reserve" nitrogen content
and growth rate such that

\[ \mu = \mu_{\text{max}} \left( \frac{Q^1}{A + Q^1} \right), \]

where \( A \) is a growth constant being that concentration of "reserve" nitrogen required to produce a growth rate of \( \mu_{\text{max}}/2 \). This means that the observed growth rate (\( \mu \)) and \( K_S \) observed in laboratory experiments are a function of the history of the inoculum (i.e. "excess nutrient status") in addition to the nitrogen content of the new environment. Examples of this phenomenon in relation to other nutrients are given in the reports of Eppley and Strickland (1968) and Droop (1968, 1970, 1973).

3.3.1 Nitrate reduction

While ammonia is probably the most readily utilized source of nitrogen by algae, many also grow on nitrate (Syrett, 1962; Naylor, 1970). That light has a stimulatory effect on nitrate reduction has been known for some time but the mechanisms involved are obscure. In *Dunaliella tertiolecta* (Grant, 1967, 1968) the assimilation of both nitrate and nitrite were stimulated twenty-fold in the presence of light and \( \text{CO}_2 \). Since organic carbon sources such as glucose, glycerol, acetate, pyruvate and 2-oxoglutarate were not effective as substitutes for \( \text{CO}_2 \), it was proposed that nitrate reduction was linked to photosynthesis and located in chloroplasts. Similar results were obtained with *Chlorella* spp., *Tetraselmis suecica* and *Phaeodactylum tricornutum*. *Haematococcus pluvialis*, however, will grow in the dark with nitrate as sole nitrogen source with acetate as source of carbon (Droop, 1961) as will *Chlamydomonas rheinhardii*, but only after a period of adaptation (Thacker and Syrett, 1972a). In this latter organism acetate would substitute for \( \text{CO}_2 \) in the light and, moreover, if cultures were allowed to accumulate an internal reserve of carbon (by growth in a nitrogen-deficient medium) then assimilation of nitrate in the dark occurred in the absence of an exogenous source of carbon.

Cultures of *Ditylum brightwellii* grown on nitrate as nitrogen source took up and assimilated this nitrogen source only in the light and nitrite reduction apparently required photosynthesis since this process was inhibited by DCMU (Eppley and Coatsworth, 1968). Cultures grown on nitrite, however, took up nitrate in light or dark but nitrate absorbed
Nitrate reduction in nitrite grown cells occurred readily in the light and was inhibited by DCMU and since in these cultures of nitrite grown _Ditylum_ the content of NADH nitrate reductase was low, Eppley and Coatsworth suggested the presence in this organism of a photosynthetic, light-induced nitrate reductase distinct from the NADH-linked system. Such a system has also been reported to occur in _Dunaliella tertiolecta_ (Grant, 1968).

Eppley _et al._ (1970) assayed the NADH-dependent formation of nitrite from nitrate in extracts of Peru Current phytoplankton and found that enzyme activity showed diel periodicity with maximum activity near noon and minimum activity near midnight. The decline in activity after dark was complete in 5 h. They proposed that enzyme synthesis occurred only in light and in a control experiment demonstrated that nitrate reductase did not develop in samples held in darkness during the hours of daylight. Also due to this light requirement for enzyme synthesis, nitrate reductase activity was low at 1 per cent light depth relative to the activity recorded near the surface. Diel periodicity of this type was also shown to occur in the rates of both nitrate and ammonia assimilation in the Sargasso Sea off Bermuda (Goering _et al._, 1964) in the synthesis of nitrite reductase in _Chlorella pyrenoidosa_ (Knutsen, 1965) and in experiments with _Skeletonema costatum_ (Packard _et al._, 1971; Eppley _et al._, 1971) in which assimilation was studied in nitrogen-limited chemostat cultures equipped with light/dark cycles. In contrast, cultures of _Coccolithus huxleyi_ assimilated both nitrate and ammonia at rates sufficient to maintain low culture medium levels during both light and dark cycles. The activity of nitrate reductase, however, was higher in light than dark, indicating light dependence in nitrogen assimilating capacity (Eppley _et al._, 1971). In the North Pacific the assimilation of nitrate and ammonia in surface waters was stimulated threefold by light (Hattori and Wada, 1972) and was closely related to the activity of the phytoplankton (Hattori and Wada, 1972; McCarthy and Eppley, 1972).

### 3.3.2 Ammonia assimilation

As discussed below, ammonia is often a preferred nitrogen source to ammonia and nitrate is reduced to ammonia before being converted to organic nitrogen. There is little information available regarding the
conversion of ammonia to organic nitrogen although this is thought to occur by "reductive amination" mediated by glutamate dehydrogenase. As yet, however, there has not been a systematic search for other aminating systems such as glutamine synthetase/glutamate synthase which is widespread in bacteria and present in some yeasts (Brown et al., 1973a; Brown et al., 1974). In a nitrogen-limited environment then the system responsible for ammonia assimilation must have a very high substrate affinity (low $K_M$ for ammonia), or the cell must be capable of concentrating ammonia or there must be a large excess of an enzyme with a relatively low substrate affinity (high $K_M$ for ammonia). Glutamate dehydrogenase from many sources has a relatively high $K_M$ for ammonia although it may be that the algal enzyme has different characteristics (see Brown et al., 1974). *Chlorella* spp. may contain both NADPH-linked and NADH-linked glutamate dehydrogenase (Morris and Syrett, 1965; Kretovitch et al., 1970; Talley et al., 1972) while an NADPH-linked enzyme was reported to occur in *Ditylum brightwellii* (Eppley and Rogers, 1970). In the latter organism there is evidence that ammonia may be concentrated within the cell to a concentration of 5–10 mM and nitrogen starvation usually results in highest enzyme activities in this organism, in *Chlorella vulgaris* (Morris and Syrett, 1965) and *Biddulphia* (Lui and Roels, 1972). Glutamate dehydrogenase activity linked to NADH and NADPH was detected in enriched marine phytoplankton samples (McCarthy and Eppley, 1972) but either singly or as a sum these activities compared poorly with the rate of ammonia uptake in this system as measured with $^{15}$N. There are reports of a diel periodicity for glutamate dehydrogenase activity in *Chlamydomonas rheinhardii* (Kates and Jones, 1967) and in *Skeletonema costatum* (Eppley et al., 1971). In the latter organism glutamate dehydrogenase activity (and nitrate reductase) was maximal at the beginning of the light period.

3.3.3 Influence of ammonia on nitrate assimilation

In general the presence of ammonia in appreciable concentrations decreases or completely inhibits the utilization of nitrate and nitrite and the synthesis of nitrate and nitrite reductases. Pratt and Fong (1940) reported that *Chlorella vulgaris* utilized ammonia in preference to nitrate while Cramer and Myers (1949) showed that nitrate assimilation in *Cl. pyrenoidosa* was decreased markedly in the presence of ammonia or
by carbon starvation. In *Chlorella vulgaris* (Syrett and Morris, 1963) the assimilation of nitrate was completely inhibited by addition of low concentrations of ammonia and thus inhibition was not relieved until this ammonia had been assimilated. Ammonia, however, only partially inhibited the assimilation of nitrite, indicating that the main site of inhibition was in the reduction of nitrate to nitrite. Since ammonia had little effect on carbon-starved cells it was proposed that some product of ammonia assimilation rather than ammonia *per se* was responsible for the inhibition of nitrate assimilation. In cell-free extracts (Morris and Syrett, 1963) an ammonium sulphate concentration of $3 \times 10^{-2} \text{M}$ had no effect on the activity of nitrate reductase, although this was some thirty times higher than the concentration required to prevent nitrate assimilation by intact cells. Cultures grown on ammonia contained only low levels of nitrate reductase but enzyme activity increased rapidly on transfer to a medium containing nitrate and this stimulation was only partly inhibited by chloramphenicol and $p$-fluorophenyl alanine. While nitrate stimulated the development of nitrate reductase its presence was not obligatory for the appearance of enzyme activity since nitrogen-starved cells showed low levels of activity. Morris and Syrett (1965) extended this observation by showing that nitrate reductase activity of nitrogen-starved cells (previously grown on nitrate or ammonia) was lost if the starvation was prolonged. Many of these effects on levels of enzyme activity in cell-free extracts may be due to enzyme activation and inactivation as well as or in place of control of enzyme synthesis (Losada *et al*., 1970; Vennesland and Jetschmann, 1971; Solomonson and Vennesland, 1972; Monerno *et al*., 1972).

In *Chlamydomonas rheinhardii* (Thacker and Syrett, 1972a, b) the assimilation of nitrate was inhibited by addition of either ammonia or nitrite and the assimilation of nitrate by ammonia. As in *Chlorella* spp. ammonia-grown cells contained little nitrate reductase activity but this appeared rapidly when such cells were incubated in the presence of nitrate. Nitrate reductase activity of cells grown on nitrate declined fairly rapidly when CO$_2$ fixation was prevented in the dark, in the absence of CO$_2$ or in the presence of DCMU. There is evidence that as in *Chlorella vulgaris*, nitrate reductase from *Chlamydomonas rheinhardii* is subject to activation-inactivation reactions. In both organisms the active form of the enzyme is produced on oxidation (Jetschmann *et al*., 1972; Losada *et al*., 1973). In *Chlamydomonas* it is suggested that am-
Ammonia promotes the conversion of the active form of the enzyme to its inactive form by an indirect reduction of the enzyme itself. This is presumed to occur because ammonia acts as an uncoupler of oxidative phosphorylation and this causes a rise in the reducing potential of the cell. This process is reversible since when ammonia is removed the enzyme is oxidized and activity is restored.

Prochazkova et al. (1970) have reported that ammonia is utilized in preference to nitrate in natural freshwater plankton populations. Proctor (1957) showed that ammonia was assimilated at about twice the rate of nitrate in Haematococcus pluvialis. In Ditylum brightwellii (Eppley and Rogers, 1970) NADH-linked nitrate reductase was induced by nitrate and repressed by ammonia while nitrite reductase was induced by nitrate or nitrite and repressed by ammonia. The activities of both nitrate and nitrite reductases decreased in the absence of their respective substrates. In Biddulphia aurita (Lui and Roels, 1972) cultures grown on nitrate contained both nitrate and nitrite reductase activities, those grown on nitrite contain little nitrate reductase and those grown on ammonia little nitrate or nitrite reductase. There are a number of reports of preferential utilization of ammonia in natural marine populations of phytoplankton, for example Eppley et al. (1969b) showed that nitrate reductase is repressed by growth on ammonia but only if the ammonia concentration is high (5–15 × 10^{-6} M). Nitrate reductase, however, was synthesized during the assimilation of ammonia if the ammonia concentration was no higher than 0.5 to 1.0 × 10^{-6} M. In the samples studied nitrate reductase was present in phytoplankton netted from waters containing 2–10 × 10^{-6} M nitrate but enzyme activity was low when the nitrogen source was depleted. Eppley et al. (1970) demonstrated that the nitrate reductase activity could be correlated with the nitrate concentration of the environment. Preferential uptake of ammonia was also reported by MacIsaac and Dugdale (1972) who stated that in oligotrophic regions ammonia uptake accounted for about 80 per cent nitrogen assimilated. The presence of ammonia at concentrations greater than 0.5 × 10^{-6} M resulted in inhibition of nitrate uptake; this concentration is in good agreement with that reported earlier by Eppley et al. (1969b). Other reports of ammonia inhibition of nitrate uptake in marine phytoplankton have been made by Packard et al. (1971) and McCarthy and Eppley (1972).
3.4 **Fungi**

Microfungi are found in a variety of aquatic habitats, although their numbers are usually small in comparison with other microorganisms. The predominant aquatic fungi are the phycomycetes, although members of the other major groups, mainly represented as the unicellular yeasts, are also found. Perhaps one should qualify such a distribution pattern by pointing out that studies involving aquatic fungi are few, and the physiological investigations of these organisms are almost nil.

A general account of the fungi of oceans and estuaries has been given by Johnson and Sparrow (1961), and the concept of specific marine microfungi deserves further mention. One cannot do better than to quote from Goldstein (1963a): “Most of the Ascomycetes and Deuteromycetes recovered from the sea apparently exist there by virtue of broad salinity tolerance that also enables them to exploit other habitats. In contrast to these filamentous forms, the ocean’s lower phycomycetes are generally obligately marine and frequently stenohaline.” Further, a difference in distribution of such organisms exists between coastal waters and the open sea. Vishniac (1956, 1960) points out that whilst large populations of nonfilamentous phycomycetes exist in polluted coastal waters as free-living saprophytes in competition with bacteria, some organisms are well adapted to growth in the open ocean. The question of marine yeasts (and aquatic yeasts in general) has been considered by van Uden and Fell (1968). The available evidence would seem to suggest that the variety and numbers of yeasts are highest in inland waters with a gradation via estuaries and littoral zones to the open sea. A variety of organisms are found in different locations, but it is true to say that members of the Cryptococcaceae would appear to be predominant.

Since all fungi thus far examined are unable to fix nitrogen (see Brown *et al.*, 1974) it is apparent that the distribution of such organisms in aquatic ecosystems will be at least partially governed by the availability of the various sources of fixed nitrogen, and the ability of organisms to utilize them.

### 3.4.1 Nitrate reduction

Since nitrate is probably the most important source of fixed nitrogen in nonpolluted waters (see section 1), the ability to utilize this substrate
will be of prime importance in the determination of fungal growth in such environments. The limited capacity for nitrate utilization probably partially accounts for the limited number of different fungal types found in aquatic habitats.

Goldstein (1963a, b, c) investigated four species of the genus *Thraustochytrium* isolated from water and none were able to utilize nitrate as sole source of nitrogen. A similar result had been obtained for the monocentric chytrid *Rhizophydium*, although a related organism, *Phlyctochytrium*, had been shown to be able to utilize nitrate as sole source of nitrogen (Goldstein, 1960). Subsequent studies involving the aquatic phycomycetes *Aqualinderella fermentans* (Held, 1970) and *Althornia crouchii* (Alderman and Jones, 1971) again revealed an inability to utilize nitrate. These findings lend support to the proposition of Vishniac, mentioned earlier, that large phycomycete populations are probably not found in aquatic habitats where ammonia, or organic nitrogen, is not the main source of nitrogen available.

It is interesting that the majority of organisms listed by van Uden and Fell (1968) as having been isolated from freshwater, estuarine and marine locations are nitrate negative according to Lodder (1971). Ahearn *et al.* (1962) carried out a comparative study of marine and terrestrial stains of the red pigmented yeast *Rhodotorula*, and found that both groups were heterogeneous with respect to nitrate utilization. These findings would appear to indicate that a proportion, at least, of "aquatic" yeasts are in fact dependent upon ammonia or an organic source of nitrogen for growth and would, therefore, be at a selective disadvantage in nonpolluted conditions.

Almost nothing is known of the kinetics of uptake of nitrate in aquatic fungi; obviously such data are long overdue. Similarly there is almost total ignorance regarding the enzymology and physiology of nitrate reduction in such organisms. Recently Rivas *et al.* (1973) have characterized the nitrate reducing system from *Torulopsis nitratophila* finding a $K_M$ value for nitrate of $2.3 \times 10^{-4} \text{ M}$ and for nitrite of $1.9 \times 10^{-5} \text{ M}$. Obviously the rate-limiting concentration will be that of nitrate, and since concentrations of this ion in most aquatic environments fall below the $K_M$ value quoted above, organisms must concentrate nitrate intracellularly for efficient reduction to occur. So far as physiological considerations are concerned, it is tempting to speculate as to the possible effects on aquatic microfungi on the basis of results obtained with other nonaquatic organisms. In the absence of data relating
specifically to aquatic fungi, this temptation will be resisted, except to point out that if other fixed nitrogen sources were to exhibit an effect upon the synthesis or activity of the enzyme system, then one might expect to observe differing effects in organisms living in polluted inland or littoral waters, compared with those present in the open sea.

3.4.2 Ammonia assimilation

Although almost all yeasts (including aquatic strains) are able to utilize ammonia as sole nitrogen source, this does not appear to be the case with phycomycetes. The four strains of *Thraustochytrium* examined by Goldstein (1963a, b, c) utilized ammonia very poorly, if at all, whilst *Rhizophydium* and *Phlyctochytrium* (Goldstein, 1960) were able to utilize ammonium sulphate. Two other phycomycetes that appear not to grow using ammonia as sole source of nitrogen are *Aqualinderella fermentans* and *Althornia crouchii* (Held, 1970; Alderman and Jones, 1971). All of the above mentioned phycomycetes preferred, if not required, an organic source of nitrogen, e.g. glutamate, for growth. Although based on data from a limited number of organisms, these results would seem to emphasize the saprophytic nature of the majority of phycomycetes isolated from aquatic environments.

Almost nothing is known of the enzymology of ammonia assimilation by phycomycetes. An NAD-linked glutamate dehydrogenase has been isolated from *Blastocladiella emersonii* (LeJohn and Jackson, 1968; Sanner, 1972) but this enzyme, which is subject to an array of control mechanisms (see Brown et al., 1974), probably functions catabolically especially in view of its high $K_M$ value for ammonia. From the viewpoint of the low concentrations of ammonia present in most non-polluted waters, it would be of great interest to know whether the GS/GOGAT couple were present in phycomycetes in view of the ammonia-scavenging role attributed to this system.

The GS/GOGAT couple has been detected in some yeasts (see Mechanisms) but the organisms in which it has been found, i.e. *Schizosaccharomyces* spp., *Saccharomycodes ludwigii* are not common in aquatic habitats. Although little work has been done on ammonia assimilation specifically in aquatic yeasts, the major mechanism in the majority of yeasts is well characterized as being NADP-linked (biosynthetic) GDH. The $K_M$ of this enzyme for ammonia in the majority of yeasts examined is in the range $10–25 \times 10^{-3} \text{M}$, which raises the
question of the efficiency of its functioning at low ammonia concentra-
tions such as one would expect to find in the majority of aquatic
habitats. In most yeasts grown at such low ammonia concentrations
high levels of NADP-linked GDH are detected (Brown and Johnson,
1970; Brown and Stanley, 1972; Burn et al., 1974; Johnson and Brown,
1974) and indeed this seems to be also a property of higher fungi
(Barratt, 1963). Not surprisingly, perhaps, nitrate grown yeasts also
show high NADP-linked GDH levels (Burn et al., 1974). Such high
GDH levels are probably necessary to compensate for a high $K_M$ for
ammonia in order to utilize the substrate efficiently at low concentra-
tions. If the same yeasts are grown under conditions of ammonia excess,
then the synthesis of GDH is repressed and the levels of the enzyme fall.
However, in the majority of aquatic ecosystems one would expect the
synthesis to be continually de-repressed, and the necessary synthesis of
large quantities of enzyme in an environment which might well be
nitrogen limited would obviously place the organisms at a selective
disadvantage from the point of view of nitrogen economy.

In some yeasts the presence of glutamate and other organic nitro-
genous sources repress the synthesis of NADP-linked GDH (see Burn
et al., 1974) but induce the synthesis of “degradative” NAD-linked
GDH; however, except under highly polluted conditions, the concentra-
tions of such compounds would be expected to have little significant
effect upon the synthesis of the enzyme.

Almost nothing is known of the kinetics of uptake of ammonia by
aquatic fungi and it would be dangerous to extrapolate data obtained
with other organisms, in view of the often large differences in $K_S$ values
obtained for different substrates when aquatic and nonaquatic micro-
organisms are compared.

4 Conclusions

Nitrogen, as an important component of many biomolecules, is essential
to life. Thus, in order even partially to understand the microbial growth
characteristics of a particular habitat, a knowledge of (a) the sources of
nitrogen available in a particular ecosystem, and (b) the utilization of
particular sources by particular organisms is necessary. However,
having stated this, one must be aware of the pitfalls of oversimplification.
Nitrogen is only one of an array of interlocking factors, both chemical
and physical, which apply selective pressures to an environment; the
resultant of such parameters is the nature, qualitative and quantitative, of the microbial population in that habitat. The situation with respect to nitrogen availability in a particular location is complicated by the forms in which nitrogen may be available, and the constant and very often rapid transformation of such compounds, which results in the “nitrogen cycle”.

Thus a consideration of the mechanisms by which prokaryotic and eukaryotic microorganisms utilize available nitrogen (principally in an inorganic form) and the physiological or ecological implications of modulation of such mechanisms by environmental parameters, both chemical and physical, is a positive approach to one aspect of a complex problem. We believe that provided one is aware of the limitations and constraints, mentioned above, on such an approach, then interpretation of observed data (mainly laboratory) together with cautious extrapolation has an important role to play in basic understanding of natural ecosystems.

The sources of nitrogen available, and the cycling of such sources, are important in aquatic environments especially from the point of view of possible pollution and subsequent eutrophication (which we shall define as “an alteration in ecofactors, either physical or chemical, which results in the relief, either short or long term, of the growth constraining parameters of certain organisms within that environment”). It is pertinent, therefore, to ask whether nitrogen sources are ever growth limiting in such aquatic habitats. It would appear unlikely that under most conditions dinitrogen concentration would limit nitrogen fixation. However, in the cases of nitrate, nitrite and ammonia it is conceivable that their respective concentrations could limit the growth of those microorganisms that are able to utilize them. In this context it is relevant to consider those situations where relief of limitation may take place. With respect to nitrate and (to a much lesser extent) nitrite availability these include upwelling of water from lower regions of higher concentration, the exogenous addition of such ions (pollution) and the numbers and activity of nitrifying organisms. With respect to raised ammonia levels, these will probably be due to either numbers and activity of denitrifying organisms, or to the input of polluting mixtures, where most of the ammonia available may well be due to microbial deamination of nitrogenous organic compounds. Thus, in the case of non-nitrogen-fixing microorganisms in regions where the rate of supply of fixed inorganic sources of nitrogen may be limiting, growth of such organisms
could well be stimulated by changes, either chemically or physically mediated, in the concentrations of these substrates. Such relief is more likely to be observed on a large scale in inland, estuarine and coastal waters, especially near centres of population density, rather than in the open sea. One should be cautious of “false negatives”; the finding that the concentration of a particular N source in a particular environment is low could well reflect a short half-life, viz. rapid production and rapid utilization of the compound.

The concentrations of the various fixed inorganic nitrogen sources are usually very low and although, as discussed above, ammonia is generally taken up in preference to nitrate in laboratory cultures of bacteria, algae and fungi, nitrate must be considered to be the principal source since in most instances its concentration is higher than ammonia. Having, perhaps, stated the obvious it is pertinent to remark that ammonia is subject to much more rapid turnover than nitrate and the concentrations found in many environments may not be an adequate reflection of its significance as an assimilatory nitrogen source. Due to the very low substrate concentrations found in aquatic environments it is significant that very high substrate affinities for uptake and assimilation are evident in those instances where these have been measured. A consideration of the relative contribution of fixed sources of nitrogen is obviously of little significance in environments which are nitrogen limited and, moreover, there is evidence showing that different nitrogen sources may be taken up simultaneously. For example, natural populations of marine phytoplankton take up nitrate and ammonia at the same time if the ammonia concentration does not exceed 0.5 to $1.0 \times 10^{-6}$ M, *Azotobacter chroococcum* utilizes dinitrogen and ammonia at the same time and nitrate and ammonia are utilized simultaneously by a marine pseudomonad, but only when the ammonia concentration is low. It is not apparent, however, to what extent “population effects” influence the repression of dinitrogen fixation by nitrate and ammonia and nitrate utilization by ammonia and results from laboratory experiments or from field experiments in which the concentration of organisms is higher than in a natural environment must be viewed with caution until this information is available.

The control of the synthesis of some enzymes involved in inorganic nitrogen assimilation is by repression and de-repression mechanisms while others are controlled by repression and induction. Into the first category come, for example, nitrogenase in bacteria and probably in
blue-green algae, nitrate reductase in a marine pseudomonad and some fungi, glutamine synthetase in bacteria, GOGAT in some bacteria and biosynthetic GDH in many yeasts. In the absence of “population effects” it is doubtful whether the concentration of repressing sources of nitrogen are ever sufficiently high in a natural environment to repress enzyme synthesis. This may be wasteful in terms of an enzyme being synthesized and not utilized but its presence could well put the organism concerned at an advantage in a changing environment. Conversely, with those enzymes that require the presence of substrate for enzyme synthesis (and/or activity), which include algal nitrate reductase, nitrate reductase in yeasts and in some filamentous fungi and NADP-linked GDH in some bacteria, there may be instances when the concentration of the relevant substrate is insufficient to allow complete induction of enzyme synthesis.

In terms of the overall nitrogen cycle as outlined in section 1 many of the transformations result only in a turnover of nitrogeneous materials in a balanced manner without a net gain or loss to the system. The exceptions to this are dinitrogen fixation, and denitrification resulting in the production of dinitrogen. The fixation of dinitrogen is of particular significance to habitats of low fixed nitrogen content but it is apparent that for nitrogen-limited systems to exist then some constraints must operate with respect to nitrogen fixation. Such limiting factors are of crucial importance. It is unlikely that nitrogen fixation is limited by dinitrogen availability, or by the concentration of fixed nitrogen sources except in some eutrophic freshwater locations. Other factors undoubtedly influencing the nitrogen fixation rate must include macro- and micro-nutrients, light, oxygen tension, temperature and salinity. Little is known of the effect of salinity on nitrogen fixation but it is noticeable that the number of types of blue-green algae observed in saline environments are fewer than those of freshwater environments. In recent years the popular method of quantifying nitrogen fixation has involved the use of the acetylene reduction technique, the great advantage of which is convenience. It is important to evaluate the absolute correlation between rates of acetylene reduction and actual nitrogen fixation and to determine whether such a correlation holds under all environmental conditions. In addition, there needs to be comparative information on the permeability of the two substrates, on the relative $K_M$ values for nitrogenase and any possible inhibitory effects of the acetylene concentrations employed.

The identification of the limiting substrate in a given environment
at a given time is difficult to establish with certainty since a variety of nutrients may be present in near-limiting concentrations and changes in their relative proportions may change the nature of the limitation. This concept may be of particular significance in estuarine or near-shore environments where conditions of salinity and nutrient concentration may be subject to rapid change due to mixing of freshwater and sea water.

The medium salt concentration is known to increase the size of the intracellular amino acid pool of the marine diatom *Phaeodactylum tricornutum* (Besnier *et al.*, 1969) and of a number of bacteria (Tempest *et al.*, 1970; Brown and Stanley, 1972) including some “marine pseudomonads” (Stanley and Brown, 1974) and such increases may serve some osmoregulatory function. In bacteria an increased medium concentration of NaCl has a marked effect on the pool contents of glutamate and proline. In nitrogen-limited chemostat cultures the pool content of marine pseudomonads may rise from $3.3 \times 10^{-3} \text{M}$ at $0.2 \text{M} \text{NaCl}$ to $20 \times 10^{-3} \text{M}$ at $0.5 \text{M} \text{NaCl}$ while in a carbon-limited system the pool rises from $33 \times 10^{-3} \text{M}$ to $165 \times 10^{-3} \text{M}$ over a similar salt range. The marine pseudomonad PL$_1$ shows an extremely rapid response to medium salinity with the pool glutamate level doubling within 5 minutes of adding NaCl as a “pulse” in a carbon-limited system. Under nitrogen limitation the response is slower but even so the pool glutamate rises some four-fold in 100 minutes in an organism growing with a mean generation time of 414 minutes (Stanley and Brown, 1974). Whether this is *de novo* amino acid synthesis or the breakdown of an intracellular polymer is unknown. In the reverse situation, in an environment in which the salinity is decreased then there is a rapid, and selective, decrease in pool content which, in laboratory cultures, results in the excretion of significant quantities of amino acid (glutamate and proline) into the culture medium. This excretion did not account quantitatively for the decrease in pool content and it seems that while very rapid mechanisms exist for increasing the pool content with an increased salinity, then equally rapid mechanisms exist to decrease the pool content with a minimal loss of amino nitrogen by excretion.

Another interaction of salinity and nitrogen assimilation may be seen in the response of a freshwater psychrophilic pseudomonad (strain B$_4$) to growth at different salinities. As shown in Fig. 2, the salt tolerance of this organism was strongly dependent on the medium
Fig. 2. Salt sensitivity of a freshwater pseudomonad (B₄) grown in chemostat culture (10 °C, dilution rate 0·1 h⁻¹). ○, Nitrate limited; ●, nitrate excess; △, ammonia excess; □, glutamate excess.

nitrogen source. The tolerance towards salt shown by cultures grown in the presence of an excess of ammonia was also shown in those grown on glutamate (the culture medium of which contained substantial quantities of ammonia) while no such tolerance was shown by ammonia or nitrate-limited cultures or those grown in the presence of a nitrate excess. Strain B₄, in common with the marine pseudomonads and *Ps. aeruginosa* and *Ps. fluorescens*, when grown on nitrate excess behaves as if nitrogen limited. Thus salt tolerance is shown by cultures grown on ammonia excess but not by nitrogen-limited cultures and these facts may be of considerable significance in relation to the competition between freshwater and marine bacteria in a polluted estuary. Another organism (strain E₄) was able to tolerate up to 0·6 M NaCl (i.e. above marine salinities) when grown on either nitrate, ammonia or glutamate and did not show protection against NaCl in the presence of an ammonia excess, nor was such protection noticed in the marine pseudomonads which will grow in a range 0·2 to 1·0 M NaCl on nitrate, am-
monia or glutamate. It seems likely that "ammonia protection" may occur only in those organisms not "adapted" to high concentrations of NaCl.

The ability to utilize the principal source of fixed nitrogen, that is nitrate, will to some extent govern the ability to grow in an aquatic environment and this is borne out by the fact that most aquatic microorganisms are able to utilize nitrate. The majority of bacteria present in nonpolluted aquatic environments are Gram-negative and correlation may exist between the amino acid pool content and the ability to grow and survive under these conditions. Thus the Gram-positive Bacillus spp. grown in chemostats under nitrogen limitation contained, on average, ten times the amino acid pool of a range of Gram-negative bacteria grown under similar conditions. The effect of increased salinity in both Gram-negative and Gram-positive organisms was to increase markedly the amino acid pool content (Brown and Stanley, 1972); for example, B. subtilis grown in the presence of 0.375 M NaCl contained pool levels of glutamate of 130 mM and of proline 220 mM. In a nitrogen-limited environment, therefore, it might be expected that greater bacterial growth would be supported at a lower salinity than when the salt concentration is higher, and, moreover, in such environments Gram-negative organisms may be expected to predominate. It would be of interest to know whether a similar situation obtains in microalgae, and if so to what extent this may control the distribution of such organisms in saline environments.

Thus it can be seen that the nature and concentration of nitrogen sources in aquatic environments may exert effects at three levels, namely that of the cell, that of the population and that of the ecosystem, both micro and macro.

This review was completed in April 1974, since when the presence of glutamate synthase (ferredoxin linked) has been demonstrated in blue-green algae, eukaryotic algae and higher plants. For further information, the review by Miflin and Lea (1976) is recommended to the reader.

References
Alderman, D. J. and Jones, E. B. G. (1971). Physiological requirements of two


Brown, C. M. and Stanley, S. O. (1972). Environment mediated changes in the
cellular content of the "pool" constituents and their associated changes in cell physiology. Journal of Applied Chemistry and Biotechnology, 22, 363–389.


Fogg, G. E. (1949). Growth and heterocyst production in *Anabaena cylindrica*
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I. Seasonal variation and the role of heterocysts. *Limnology and Oceanography*, 17, 678-692.


